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(54) Title: STRAINS OF BACTERIOPHAGE USEFUL FOR RESCUING PATIENTS INFECTED WITH VANCOMYCIN-RESISTANT ENTEROCOCCUS FAECIUM

(57) Abstract: The present invention involves the use of specific phages, designated ENB6 and ENB13, that kill many clinical isolates of vancomycin-resistant *Enterococcus faecium* and of vancomycin-sensitive *Enterococcus faecium*. The genome of one of the phage strains, ENB6 has been partially sequenced, and is shown to not contain nucleotide sequences for known bacterial virulence genes or for the vancomycin resistance cassette. Its efficacy in rescuing mice from otherwise-fatal bacteremias is documented herein.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/06718

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :A01N 63/00; A61K 39/02

US CL : 424/93.6, 234.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/93.6, 234.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WEST, DIOALOG, MEDLINE, BIOSIS, SCISEARCH, EMBASE

search terms; vancomycin resistant enterococcus faecium, phage, bacteriophage, lytic, ENB6, ENB13, treating, antibiotic

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5,688,501 A (MERRIL et al) 18 November 1997, see entire document.	1-13

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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Strains of Bact riophag Useful for Rescuing Patients Infected With Vancomycin-Resistant Enterococcus Faecium

FIELD OF THE INVENTION

Several clinically important species of bacteria have become multidrug resistant ("MDR"). One of these is Enterococcus faecium, a commensal that does not cause disease in its habitual niche (the intestines) but which can breach the gut barrier and cause bacteremias if the immune system fails to eliminate the bacteria. Immunocompromised patients cannot eliminate these bacteria, and deaths in such patients are becoming increasingly commonplace.

As E. faecium acquired resistance to increasing numbers of antibiotics (e.g. penicillins, cephalosporins and aminoglycosides), treatment options became progressively narrowed until vancomycin was the drug of last resort. In 1989 the first clinical isolates of vancomycin-resistant E. faecium (VREF) were reported. Physicians were then confronted with a pathogen that was difficult and often impossible to treat. In recent years the prevalence of these vancomycin-resistant strains has increased to the point that hospitals typically report that approximately 40% of the E. faecium clinical isolates are vancomycin resistant. Correspondingly, fatal bacteremias are being reported in steadily increasing numbers.

While the pharmaceutical industry does introduce new antibiotics from time to time, it has become commonplace that new antibiotics become rapidly resisted by multidrug resistant ("MDR") bacteria. For example, Synercid® recently entered

the market as a treatment for vancomycin-resistant bacteria, including VREF. Resistance to this antibiotic began to appear even while it was in clinical trials, and by the time it was approved for commercial sales approximately 20% of VREF clinical isolates were reported fully resistant to this new antibiotic. The reason that MDR bacteria are so efficient at resisting newer antibiotics (even those to which they have never been exposed) is that the resistance mechanisms they've acquired enable them to defeat many different classes of antibiotics. For example, a mutant efflux pump can transport out many classes of drugs; and a mutation in the ribosomal subunit targeted by antibiotics can defeat several classes of drugs. An alternative to antibiotics is therefore needed to control such MDR bacteria.

Bacteriophage (phage) therapy offers one such alternative. The present invention describes several examples of phage strains (for example ENB6) that rescues mice from a fulminant VREF bacteremia.

BACKGROUND OF THE INVENTION

As described in US Patent No# 5,688,501 by Merril et al (and incorporated by reference herein), phage therapy of human bacterial infections failed for a number of technical reasons. One of the technical reasons was that phages tend to be rapidly cleared from the systemic circulation by the filtering action of the organs of the reticulo-endothelial system (RES). This rapid clearance prevents the phages from remaining in circulation long enough to reach and infect the target bacteria infecting the patient.

The above-cited invention solved the problem of rapid clearance by introducing a novel approach called "serial passage". In that technique, a large number of phages of a wild-type strain are injected into an animal, blood samples are taken at various intervals, and any phage particles still remaining in circulation at the time of the venipuncture will be present therein and can be grown to high titer on the host bacteria. This technique therefore selects for phage variants whose surface coat proteins are not readily detected by the RES, and such variants are amplified by cloning at the end of each round of serial passage. Since the phages being selected must be able to produce plaques on the lawn of the host bacteria, the technique also selects for those mutants that retain their ability to lyse the target bacteria. Finally, the long-circulating phage mutants obtained thereby were superior to the wild-types from which they were derived, in terms of rescuing an animal from an otherwise-fatal bacteremia. In the above-referenced patent, the bacterial target was a strain of E. coli, and the wild-type phage strain used was lambda coliphage.

In the present invention, phage stains that attack VREF hosts have been discovered by the present inventors. These strains were discovered through screening samples of sewage from the waste management system of Montgomery County, Maryland.

SUMMARY OF THE INVENTION

Phage strains were grown by standard techniques known in the art, by plating them on clinical isolates of VREF which were obtained from hospitalized

patients (with no identifiers as to the name of the patients). These stains are lytic when propagated in many clinical isolates of VREF.

These phage strains were grown to high titer, and they were characterized and defined through the methods described below using the phage strain ENB6 as an example.

DETAILED DESCRIPTION OF THE INVENTION

Details on the characterization of and host range of phage ENB6 are provided in this section. Details on the phage's utility, in terms of rescuing animals from an otherwise-lethal bacteremia, are provided in the section that follows.

1. Genomic sequencing

50 mg of phage ENB6 DNA was sheared and then random fragments were "shotgun cloned" into an M13-based vector for sequencing. The raw data was pre-screened and then the individual sequences were compiled into overlapping contigs.

The ENB6 genome contains at least 120 kb of DNA as determined by sequencing and gel electrophoretic analyses of extracted DNA. A total of 94.4 kb of nucleotide sequence has been defined at 99% confidence, while 24.7 kb has

been defined at a lower level of confidence. The remaining amount is presently undefined.

2. Analyzing the phage's genome for nucleotide sequences of interest, using homology searches on databases as well as PCR probes

The ENB6 nucleotide sequences have been compared to all genes and proteins registered in the databases using two alignment algorithms, BLASTN (nucleotide sequence comparisons) and BLASTX (putative amino acid sequence comparisons). All alignments of high confidence matched genes and gene products of other bacteriophages including those for head, tail, polymerase and lysin proteins. No extensive and significant match was found at the nucleotide or predicted protein level to recognized whole genes of bacterial factors for pathogenicity, infectivity, invasion, attachment or antibiotic resistance. However, four short and dispersed alignments to these kinds of undesirable factors were found as shown in Figure 1 and Table 1. The fraction of each protein exhibiting some similarity to a potential gene product from ENB6 is not greater than 30 % in any example, meaning, at best, only a partial gene exists. The short lengths of identity suggest that only a subtle similarity exists at the amino acid sequence level. If actually translated into protein products, these fragmented domains would either be not functional or unfamiliar.

Thus, we find no evidence of whole genes for potentially hazardous factors in the known nucleotide sequence of phage ENB6. Understanding that only part of

Table 1. Undesirable proteins found by BLASTX alignments of th oretical proteins derived from ENB6 nucleotide sequence.

Source of query sequence	Target protein found to have some alignment	Alignment scores: identity per length (%), gaps per length	Fraction of target aligned
Contig 34	**Plasminogen binding protein (class C *Streptococci*)**	**32/108 (29%), 13/108**	**61/454 (13 %)**
Contig 37	**Orf1 protein of insertion element IS232**	**14/30 (46%)**	**29/431 (7 %)**
Contig 43	**Hemagglutinin (Influenza A virus)**	**17/41 (41%)**	**116/566 (20 %)**
Contig 49	**orf14 protein of transposon Tn916**	**37/124 (29%), 6/124**	**96/329 (29 %)**

the genome was screened by database searches, we have undertaken a second approach to inspecting the ENB6 phage for potentially undesirable genes. We have designed oligonucleotide primers for physical screening of the phage DNA by PCR amplification. The genes searched are listed in Table 2.

Thus we have used sequence alignment searches and physical tests for known genes to address the concern for a potential risk of horizontal gene transfer through the therapeutic use of bacteriophage phage ENB6.

3. Electron microscopic study

Figure 2 is an electron microscopic picture of phage ENB6.

The routes of administration include but are not limited to: oral, aerosol or other device for delivery to the lungs, nasal spray, intravenous, intramuscular, intraperitoneal, intrathecal, vaginal, rectal, topical, lumbar puncture, intrathecal, and direct application to the brain and/or meninges. Excipients which can be used as a vehicle for the delivery of the phage will be apparent to those skilled in the art. For example, the free phage could be in lyophilized form and be dissolved just prior to administration by IV injection. The dosage of administration is contemplated to be in the range of about 10^3 to about 10^{13} pfu/per kg/per day, and preferably about 10^{12} pfu/per kg/per day. The phage are

Table 2. Proteins screened by PCR amplification of ENB6 DNA.

Genes Targeted for amplification by PCR	Source and Description
cylL1, cylM, cylB, cylA	**Cytolytic genes contained on the conjugative (transferable) plasmid pAD1 of *E. faecalis*.**
traC	**Hemolytic bacteriocin from pAD1 of *E. faecalis*.**
pneu	**Pneumolysin from *S. pneumonae*.**
sly	**Cytolytic toxin from *Streptococcus suis*.**
slo	**Streptolysin O from plasmid pMK157 *Streptococcus cannis*.**
slo	**Streptolysin O from group A, C and G *Streptococci*.**
sagC	**Streptolysin S**
L50	**Enterocin L50 from *E. faecium*.**
aph	**Resistance to aminoglycoside antibiotics (gentamycin, kanamycin) from *E. casseliflavus*.**
genta	**Newly characterized resistance gene to gentamycin from *S. aureus*.**
ermAM	**Resistance to erythromycin from plasmid pAM-b-1 of *S. faecalis*.**
ery	**Resistance to erythromycin from transposon Tn917.**
penA	**Class AmpC b-lactamase from *S. pneumoniae* giving resistance to penicillin antibiotics.**
orf14	**Orf14 protein of transposon Tn916 of *E. faecalis*.**
orf1	**Orf1 protein of insertion sequence (mobile DNA element) IS232 of *B. thuringiensis*.**
tetM	**Tetracycline Resistance from transposon Tn916 of *E. faecalis*.**
esp	**Surface protein of virulent *Enteroccoci* clinical isolates.**

administered until successful elimination of the pathogenic Enterococcus faecium is achieved.

As used in the present application, the term "substantially reduce" indicates that the number of bacteria is reduced to a number which can be completely eliminated by the animal's defense system or by using conventional antibacterial therapies.

The present invention will be particularly useful in treating critically ill patients or those with severe underlying disease or immunosuppression (e.g. patients in ICUs or in oncology or transplant wards), patients who have had an intraabdominal or cardio-thoracic surgical procedure or an indwelling urinary or central venous catheter, and persons who have had a prolonged hospital stay or received multi-antimicrobial and/or vancomycin therapy.

Deposits of ENB6 (ATCC # PTA-40) and ENB13 (ATCC # PTA-39) were made on May 12, 1999 at the American Type Culture Collection, 10801 University Blvd., Manassas, VA. 20110-2209.

The foregoing embodiments of the present invention are further described in the following Examples. However, the present invention is not limited by the Examples, and variations will be apparent to those skilled in the art.

EXAMPLES

1. VREF Bacteremia Rescue Experiment #1: Dose-Finding Study

Figures 3 and 4 show the results of a dose-finding study.

Materials and Methods:

We had previously determined that the $2xLD_{50}$ dose for a clinical VREF isolate designated CRMEN44 is 1×10^9 CFU, when injected I.P. into one month-old balb/c female mice. In other studies (data not shown here), we had determined that the I.P. injection of this bacteria strain causes a bacteremia within 15 minutes, and that the I.P. injection of phage ENB6 causes a viremia within 15 minutes. In this study, the following dosages of phage ENB6 were administered once (and only once) I.P., exactly ½ hour after the bacterial challenge: 3×10^9, 3×10^8, 3×10^6, and 3×10^4 PFU plaque forming units (PFU). In addition, a dose of 3×10^9 PFU was administered I.P. to another set of animals, as a control, with no bacterial challenge.

The non-parametric rating scale for observable signs of illness is as follows: 5 = Normal animal; 4 = Mild lethargy; 3 = Mild lethargy + Ruffled fur; 2 = the above, plus exudate around the eyes; 1 = Moribund; and 0 = Dead.

Results:

Phage administered as a control did not produce any detectable symptoms in the animals. Bacteria administered without any phage treatment caused the death of all the animals, within 48 hours. With the two highest dosages of phage there were

no deaths, and the animals recovered within 24 hours from the minimal signs of illness that had developed, with no relapse over a period of 21 days of observation. While there were some deaths with the two lowest dosages of phage, nevertheless roughly half the animals in these groups survived (and recovered completely) after becoming moderately ill.

Discussion:

Phage ENB6 rescues animals from an otherwise-fatal dose of VREF, a bacterial pathogen for which no consistently reliable antibiotic is currently available. The infection here is fulminant, using a concentration of bacteria (10^9, which will be very concentrated in the 3 ml of blood in a mouse's circulatory system) that is orders-of-magnitude greater than that found in bacteremic humans (where titers in blood reach only 10^2 to 10^4 CFU per cc).

Conclusion:

While an IND approval will be required from the FDA before such phages can be administered therapeutically to humans, phage strains that lyse VREF hosts *in-vitro* should be able to kill the bacterial targets wherever encountered (*in-vivo*), whether within the mouse or the human circulatory system. Moreover, multiple phage doses will be employed in treating humans. In this experiment only one dose was administered, in order to demonstrate the ability of the phages to grow exponentially in number and to thereby overwhelm the target bacteria.

2. VREF Bacteremia Rescue Experiment #2: Delayed Treatment

Figures 5 and 6 show the results of delay in the treatment of a fulminant bacteremia.

Materials and Methods:

Same as in Experiment 1, except for the dosage and timing of the phage administration. In this experiment, only the highest dose (3×10^9) of phage ENB6 was administered. After the I.P. bacterial challenge, the one (and only one) I.P. administration of the phage dose was delayed until one or another of the following time points: 2, 5, 8, 14, 18 and 24 hours. One group of animals received no phage treatment, as a control.

Results:

With no treatment, all animals were dead within 48 hours. With treatment delayed 2 hours and 5 hours, all animals survived (after becoming moderately ill). With treatment delayed from 8 – 24 hours approximately half the animals died, but for the half that survived, even though the degree of illness reached was severe, nevertheless there was full and complete recovery by day 4 or 5, with no relapse.

Discussion:

Even when treatment of a fulminant bacteremia in mice is delayed, phage ENB6 tends to rescue the animals from an otherwise-fatal dose of VREF. The rescue is

100% with delays up to and including 5 hours. With delays between 8 – 24 hours, approximately 50% of the animals survive and go on to recover completely.

Conclusion:

While an IND approval will be required from the FDA before such phages can be administered therapeutically to humans, phage strains that lyse VREF hosts *in-vitro* should be able to kill the bacterial targets wherever encountered (*in-vivo*), whether within the mouse or the human circulatory system. In the human, concentrations of VREF are orders-of-magnitude lower than the concentrations achieved here, so it should be that much easier to achieve a therapeutic success. Moreover, in treating humans, multiple administration of phage will be employed. In this experiment only one dose was administered, in order to demonstrate the ability of the phages to grow exponentially in number and to thereby overwhelm the target bacteria.

We claim:

1. A wild-type phage which is lytic for strains of vancomycin-resistant *Enterococcus faecium* (VREF) as well as for strains of vancomycin-sensitive *Enterococcus faecium* (VSEF), wherein said phage is selected from the group consisting of ENB6 (ATCC# PTA-40), and ENB13 (ATCC# PTA-39)

2. A method for treating an *Enterococcus faecium* infection comprising administering an amount of a phage selected from the group consisting of ENB6 (ATCC# PTA-40), and ENB13 (ATCC# PTA-39) effective to eradicate or substantially reduce an *Enterococcus faecium* infection to a patient in need of such treatment.

3. The method according to claim 2, wherein said *Enterococcus faecium* is vancomycin-resistant *Enterococcus faecium.*

4. The method according to claim 2, wherein said phage is administered by a route selected from the group consisting of orally, topically, intravenously, intra-arterially, intraperitoneally, intrathecally, by inhalation, by nasal spray, by irrigation of a wound, by suppository, and by enema.

5. The method according to claim 2, wherein said phage is administered at a total dose of between $10^3 - 10^{12}$ PFU.

6. The method according to claim 5, wherein said phage is administered at a total dose of between $10^5 - 10^{11}$ PFU.

7. The method according to claim 2, further comprising administering an antibiotic.

8. A method for reducing the probability of an *Enterococcus faecium* colonization becoming an infection comprising administering an amount of phage selected from the group consisting of ENB6 (ATCC# PTA-40), and ENB13 (ATCC#-PTA-39) effective to reduce the probability of such colonization becoming an infection to a patient at risk for an *Enterococcus faecium* infection.

9. The method according to claim 8, wherein said phage is administered by a route selected from the group consisting of orally, topically, intravenously, intra-arterially, intraperitoneally, intrathecally, by inhalation, by nasal spray, by irrigation of a wound, by suppository, and by enema.

10. The method according to claim 8, wherein said phage is administered at a total dose of between $10^3 - 10^{12}$ PFU.

11. The method according to claim 10, wherein said phage is administered at a total dose of between $10^5 - 10^{11}$ PFU.

12. A pharmaceutical composition comprising a phage selected from the group consisting of ENB6 (ATCC# PTA-40), and ENB13 (ATCC# PTA-39) in combination with a pharmaceutical carrier.

13. The composition according to claim 12, further comprising an antibiotic.

Figure 1. Alignments of hypothetical amino acid sequences predicted from ENB6 DNA with the protein databases.

BLASTX alignments of the highest score (Expect Value less than 0.005) to proteins of potentially undesirable factors are shown. "Query" = putative protein from ENB6; "Sbjct" = target protein found in database. Numbers = codon or amino acid number; {} = total length of target protein.

1. Plasminogen-binding protein MLC36 from group C Streptococcus sp.

```
Query: 70   RTWTEYLATGHVHDKNHAKQLERLSKRDISLGDVATVVDFMSRRNDGYITALIEQNSVNE
249
            R  T    T +V +K  A +LE+L   +++  D  ++VD M   ND   T   +   + +
Sbjct: 36   RLVTNMWKTQYVKEKQRADELEKLLHSEVA-DYNSLVDKMKVVNDSLQTTKRDYEEIEK
93

Query: 250  KLFNKL----GVTDKMRNEAKA---------EYEVELKQAQEEIKKLQEELAEKLQKGE*Y
393
            +L NKL      + +K++N+  +          E +++L Q   +   L+ EL ++ QK E
Sbjct: 94   ELGNKLKENQDLEEKLKNKEFSLGEALRYINELDLKLGQLNIDNIDLKHELEQEKQKAEAY
154 {454}
```

2. IstA protein homolog from *Bacillus thuringiensis* similar to ORF1 protein of insertion sequence IS232

```
Query: 871  QFAYDFAFSGYPQLAGMPPSSGQVDAPQMI 960
             QFA DF F    P +AG P + G+V+AP  +
Sbjct: 239  QFAQDFGFKVQPCIAGRPNTKGKVEAPMKL 268 {431}
```

3. Hemagglutinin protein from Influenza A virus

```
Query: 2636 NQAVLARNREFNKIQREGAYLDHLIEGLKEHLSEE--------------LENTNTLKYIE
2499
             N+ +   N +F++I++E + ++  I+ L++++ +                 LEN NT+   +
Sbjct: 398  NRVIEKTNEKFHQIEKEFSEVEGRIQDLEKYVEDTKIDLWSYNAELLVALENQNTIDLTD
457

Query: 2498 PELRVKGKPSDREMILCLSDWHIGAF----------VNNIDTGGYNYDIFR-ERLNS
2361
              E+    + + R++     D   G F          + +I  G YN+DI+R E LN+
Sbjct: 458  SEMNKLFEKTRRQLRENAEDMGNGCFKIYHKCDNACIESIRNGTYNHDIYRDEALNN
514 {566}
```

4. *Bacillus subtilis* protein similar to ORF14 of *E. faecalis* transposon TN916

```
Query: 2523 YDWGGGRTGRDPFESSPIATDCSSFVWWCFKHAGVELNGGATGMTTWSIIADTKLETIAT
2344
             Y WGG      +          DCS  V W F  AG+ L   A
Sbjct: 224  YAWGGS--------------NPETGFDCSGLVQWSFAKAGITLPRTAQEQ----------
259
```

```
Query: 2343 RGQKNSAIFDKMKAGDIIWF------RNCEHIGIYCGEGKMVACNGSGNMNESPTAGIIV
2182
            G         +  AGD+++F       +    H+GIY G G+M    N SG          I
Sbjct: 260  HGATKKISEKEATAGDLVFFGGTYEGKAITHVGIYVGNGRMFNSNDSG---------IQY
310

Query: 2181 SDMTSGYWWD  2152
          SD+ SGYW D
Sbjct: 311  SDLKSGYWRD   320  (329)
```

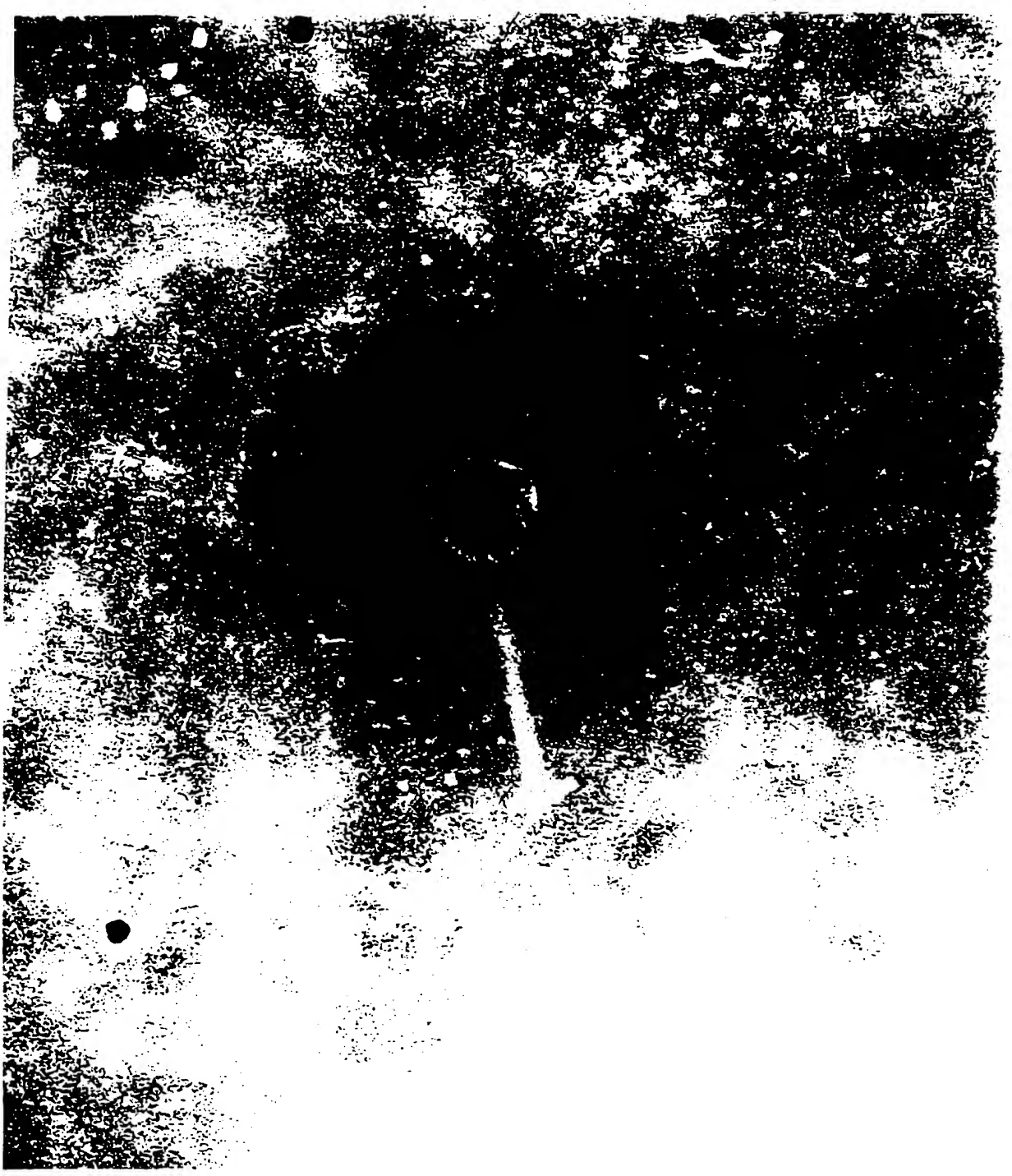


Figure 2

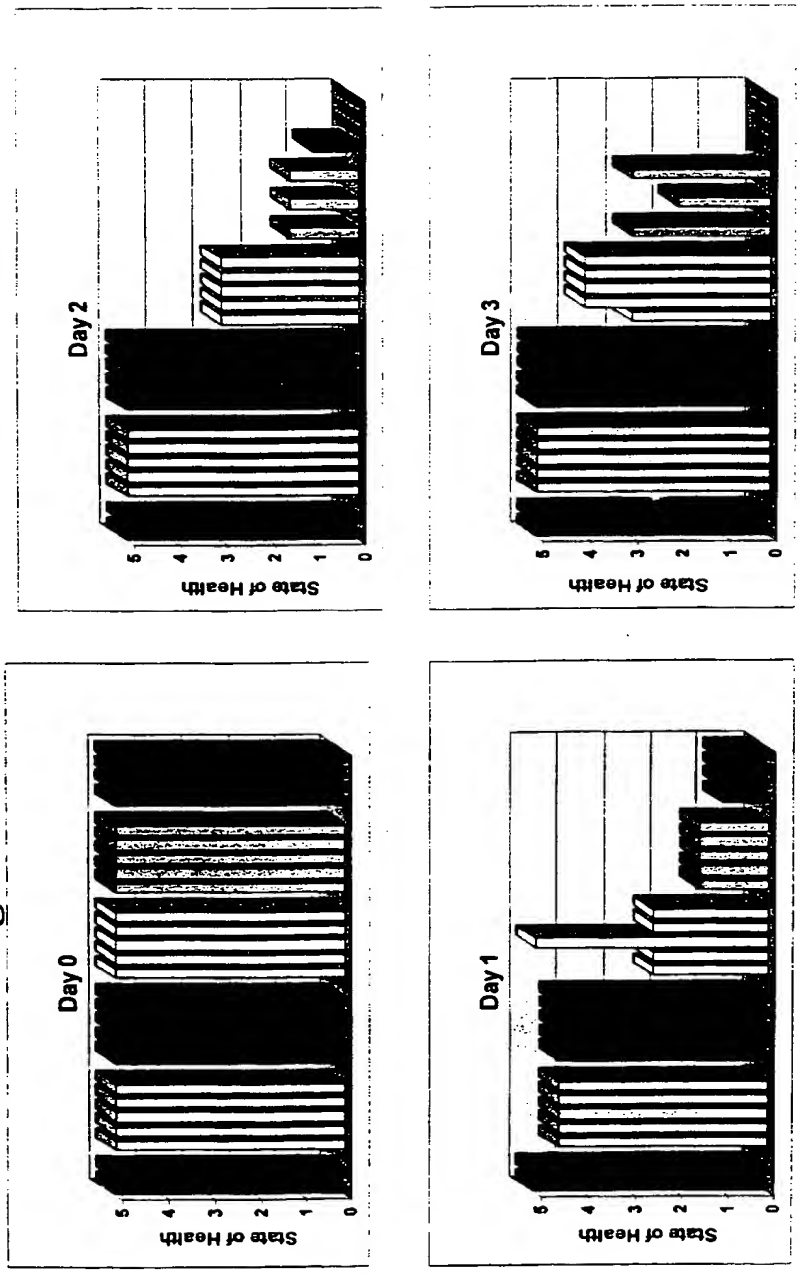
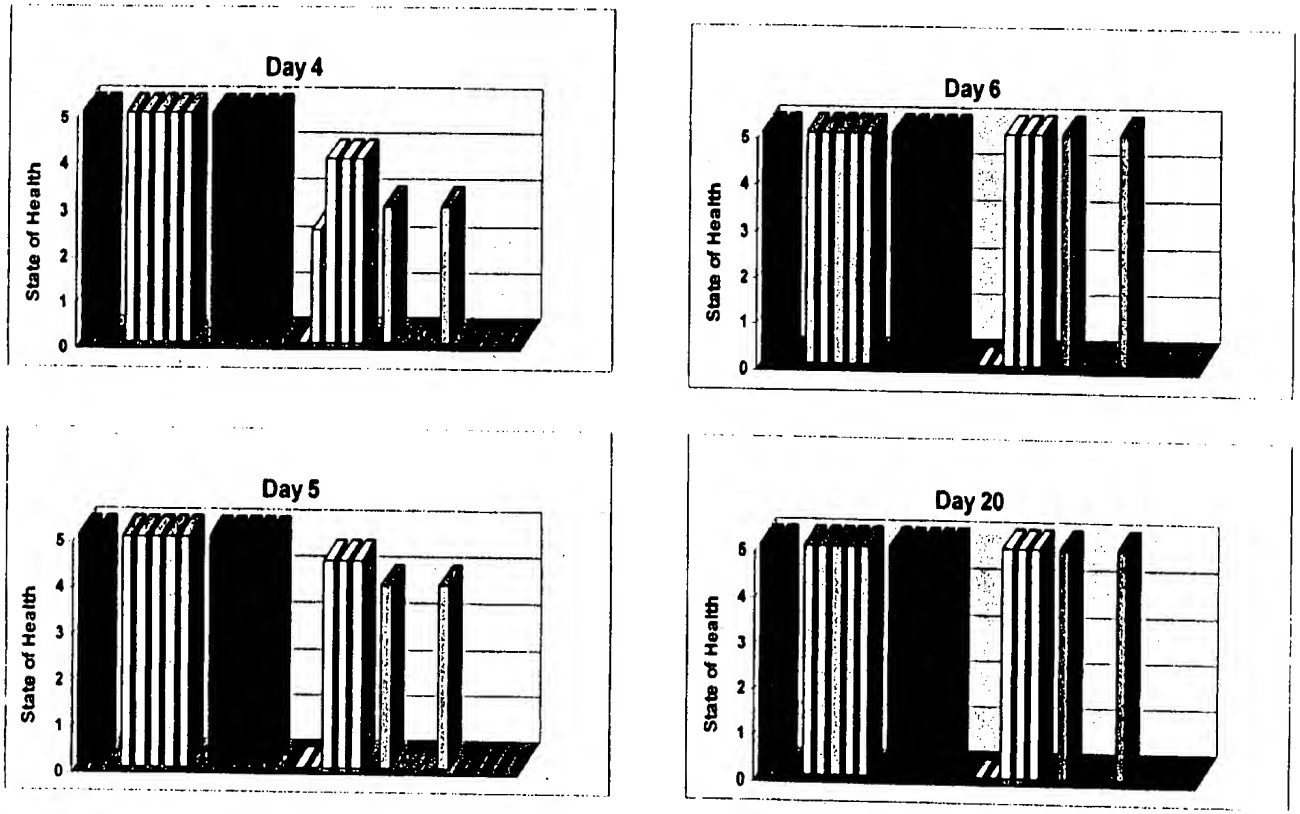

Effect of Phage Concentration on VRE Infected Mice
Day 0
Day 1
Day 2
Day 3
State of Health
0
1
2
3
4
5
Symptoms of Disease
0 => Death
1 => Moribund
2 => Ruffled fur, hunchback & closed eyes
3 => Ruffled fur & hunchback
4 => Decreased activity
5 => Normal mouse
Legend
Phage (3x10^9pfu) only
VRE (10^9cfu) + Phage (3x10^9pfu)
VRE (10^9cfu) + Phage (3x10^8pfu)
VRE (10^9cfu) + Phage (3x10^6pfu)
VRE (10^9cfu) + Phage (3x10^4pfu)
VRE(10^9cfu) only

Figure 3

Effect of Phage Concentration on VRE Infected Mice



Symptoms of Disease

0 => Death
1 => Moribund
2 => Ruffled fur, hunchback & closed eyes
3 => Ruffled fur & hunchback
4 => Decreased activity
5 => Normal mouse

Legend

- **Phage ($3x10^9$pfu) only**
- **VRE (10^9cfu) + Phage ($3x10^9$pfu)**
- **VRE (10^9cfu) + Phage ($3x10^8$pfu)**
- **VRE (10^9cfu) + Phage ($3x10^6$pfu)**
- **VRE (10^9cfu) + Phage ($3x10^4$pfu)**
- **VRE(10^9cfu) only**

Figure 4

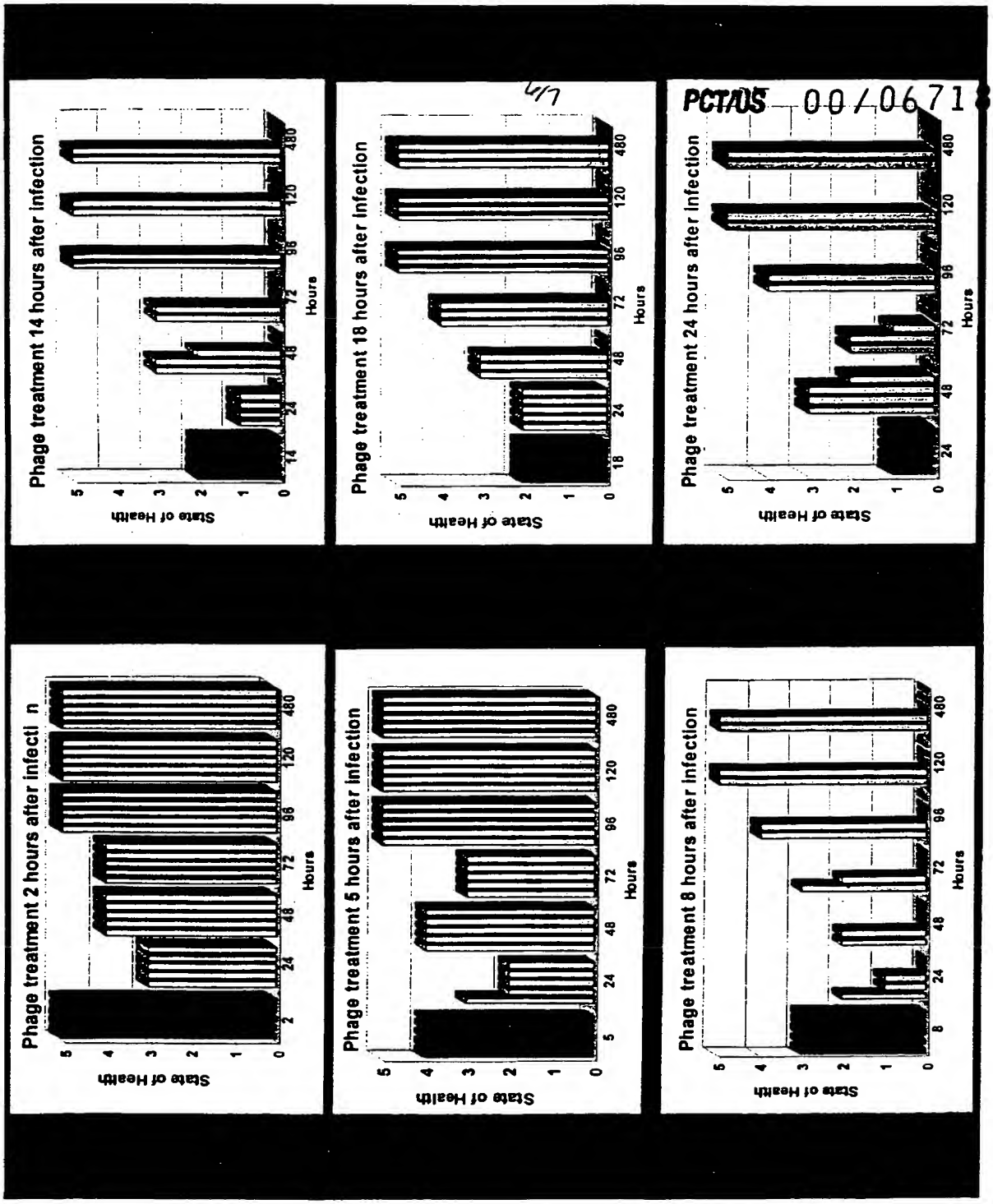

Phage treatment 14 hours after infection
Phage treatment 18 hours after infection
Phage treatment 24 hours after infection
Phage treatment 2 hours after infectl n
Phage treatment 5 hours after infection
Phage treatment 8 hours after infection
State of Health
Hours
PCT/US 00/06718

Figure 5

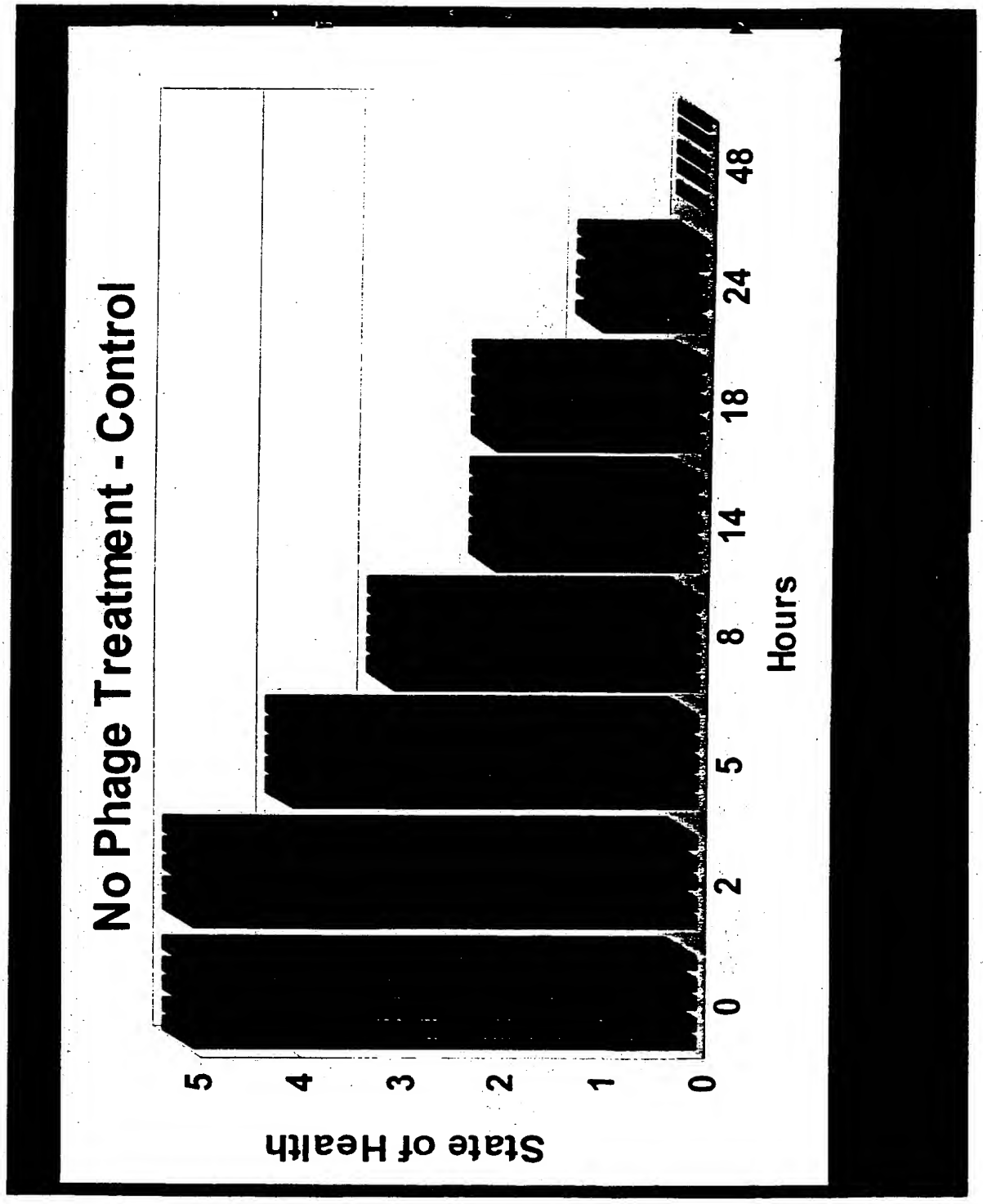

No Phage Treatment - Control
State of Health
0
1
2
3
4
5
0
2
5
8
14
18
24
48
Hours

Figure 6

Strains of Bacteriophage Useful for Rescuing Patients Infected With Vancomycin-Resistant Enterococcus Faecium

FIELD OF THE INVENTION

Several clinically important species of bacteria have become multidrug resistant ("MDR"). One of these is Enterococcus faecium, a commensal that does not cause disease in its habitual niche (the intestines) but which can breach the gut barrier and cause bacteremias if the immune system fails to eliminate the bacteria. Immunocompromised patients cannot eliminate these bacteria, and deaths in such patients are becoming increasingly commonplace.

As E. faecium acquired resistance to increasing numbers of antibiotics (e.g. penicillins, cephalosporins and aminoglycosides), treatment options became progressively narrowed until vancomycin was the drug of last resort. In 1989 the first clinical isolates of vancomycin-resistant E. faecium (VREF) were reported. Physicians were then confronted with a pathogen that was difficult and often impossible to treat. In recent years the prevalence of these vancomycin-resistant strains has increased to the point that hospitals typically report that approximately 40% of the E. faecium clinical isolates are vancomycin resistant. Correspondingly, fatal bacteremias are being reported in steadily increasing numbers.

While the pharmaceutical industry does introduce new antibiotics from time to time, it has become commonplace that new antibiotics become rapidly resisted by multidrug resistant ("MDR") bacteria. For example, Synercid® recently entered

the market as a treatment for vancomycin-resistant bacteria, including VREF. Resistance to this antibiotic began to appear even while it was in clinical trials, and by the time it was approved for commercial sales approximately 20% of VREF clinical isolates were reported fully resistant to this new antibiotic. The reason that MDR bacteria are so efficient at resisting newer antibiotics (even those to which they have never been exposed) is that the resistance mechanisms they've acquired enable them to defeat many different classes of antibiotics. For example, a mutant efflux pump can transport out many classes of drugs; and a mutation in the ribosomal subunit targeted by antibiotics can defeat several classes of drugs. An alternative to antibiotics is therefore needed to control such MDR bacteria.

Bacteriophage (phage) therapy offers one such alternative. The present invention describes several examples of phage strains (for example ENB6) that rescues mice from a fulminant VREF bacteremia.

BACKGROUND OF THE INVENTION

As described in US Patent No# 5,688,501 by Merril et al (and incorporated by reference herein), phage therapy of human bacterial infections failed for a number of technical reasons. One of the technical reasons was that phages tend to be rapidly cleared from the systemic circulation by the filtering action of the organs of the reticulo-endothelial system (RES). This rapid clearance prevents the phages from remaining in circulation long enough to reach and infect the target bacteria infecting the patient.

The above-cited invention solved the problem of rapid clearance by introducing a novel approach called "serial passage". In that technique, a large number of phages of a wild-type strain are injected into an animal, blood samples are taken at various intervals, and any phage particles still remaining in circulation at the time of the venipuncture will be present therein and can be grown to high titer on the host bacteria. This technique therefore selects for phage variants whose surface coat proteins are not readily detected by the RES, and such variants are amplified by cloning at the end of each round of serial passage. Since the phages being selected must be able to produce plaques on the lawn of the host bacteria, the technique also selects for those mutants that retain their ability to lyse the target bacteria. Finally, the long-circulating phage mutants obtained thereby were superior to the wild-types from which they were derived, in terms of rescuing an animal from an otherwise-fatal bacteremia. In the above-referenced patent, the bacterial target was a strain of E. coli, and the wild-type phage strain used was lambda coliphage.

In the present invention, phage stains that attack VREF hosts have been discovered by the present inventors. These strains were discovered through screening samples of sewage from the waste management system of Montgomery County, Maryland.

SUMMARY OF THE INVENTION

Phage strains were grown by standard techniques known in the art, by plating them on clinical isolates of VREF which were obtained from hospitalized

patients (with no identifiers as to the name of the patients). These stains are lytic when propagated in many clinical isolates of VREF.

These phage strains were grown to high titer, and they were characterized and defined through the methods described below using the phage strain ENB6 as an example.

DETAILED DESCRIPTION OF THE INVENTION

Details on the characterization of and host range of phage ENB6 are provided in this section. Details on the phage's utility, in terms of rescuing animals from an otherwise-lethal bacteremia, are provided in the section that follows.

1. Genomic sequencing

50 mg of phage ENB6 DNA was sheared and then random fragments were "shotgun cloned" into an M13-based vector for sequencing. The raw data was pre-screened and then the individual sequences were compiled into overlapping contigs.

The ENB6 genome contains at least 120 kb of DNA as determined by sequencing and gel electrophoretic analyses of extracted DNA. A total of 94.4 kb of nucleotide sequence has been defined at 99% confidence, while 24.7 kb has

been defined at a lower level of confidence. The remaining amount is presently undefined.

2. Analyzing the phage's genome for nucleotide sequences of interest, using homology searches on databases as well as PCR probes

The ENB6 nucleotide sequences have been compared to all genes and proteins registered in the databases using two alignment algorithms, BLASTN (nucleotide sequence comparisons) and BLASTX (putative amino acid sequence comparisons). All alignments of high confidence matched genes and gene products of other bacteriophages including those for head, tail, polymerase and lysin proteins. No extensive and significant match was found at the nucleotide or predicted protein level to recognized whole genes of bacterial factors for pathogenicity, infectivity, invasion, attachment or antibiotic resistance. However, four short and dispersed alignments to these kinds of undesirable factors were found as shown in Figure 1 and Table 1. The fraction of each protein exhibiting some similarity to a potential gene product from ENB6 is not greater than 30 % in any example, meaning, at best, only a partial gene exists. The short lengths of identity suggest that only a subtle similarity exists at the amino acid sequence level. If actually translated into protein products, these fragmented domains would either be not functional or unfamiliar.

Thus, we find no evidence of whole genes for potentially hazardous factors in the known nucleotide sequence of phage ENB6. Understanding that only part of

Table 1. Undesirable proteins found by BLASTX alignments of theoretical proteins deriv d from ENB6 nucleotide sequence.

Source of query sequence	Target protein found to have some alignment	Alignment scores: identity per length (%), gaps per length	Fraction of target aligned
Contig 34	**Plasminogen binding protein (class C *Streptococci*)**	**32/108 (29%), 13/108**	**61/454 (13 %)**
Contig 37	**Orf1 protein of insertion element IS232**	**14/30 (46%)**	**29/431 (7 %)**
Contig 43	**Hemagglutinin (Influenza A virus)**	**17/41 (41%)**	**116/566 (20 %)**
Contig 49	**orf14 protein of transposon Tn916**	**37/124 (29%), 6/124**	**96/329 (29 %)**

the genome was screened by database searches, we have undertaken a second approach to inspecting the ENB6 phage for potentially undesirable genes. We have designed oligonucleotide primers for physical screening of the phage DNA by PCR amplification. The genes searched are listed in Table 2.

Thus we have used sequence alignment searches and physical tests for known genes to address the concern for a potential risk of horizontal gene transfer through the therapeutic use of bacteriophage phage ENB6.

3. Electron microscopic study

Figure 2 is an electron microscopic picture of phage ENB6.

The routes of administration include but are not limited to: oral, aerosol or other device for delivery to the lungs, nasal spray, intravenous, intramuscular, intraperitoneal, intrathecal, vaginal, rectal, topical, lumbar puncture, intrathecal, and direct application to the brain and/or meninges. Excipients which can be used as a vehicle for the delivery of the phage will be apparent to those skilled in the art. For example, the free phage could be in lyophilized form and be dissolved just prior to administration by IV injection. The dosage of administration is contemplated to be in the range of about 10^3 to about 10^{13} pfu/per kg/per day, and preferably about 10^{12} pfu/per kg/per day. The phage are

Table 2. Proteins screened by PCR amplification of ENB6 DNA.

Genes Targeted for amplification by PCR	Source and Description
cylL1, cylM, cylB, cylA	**Cytolytic genes contained on the conjugative (transferable) plasmid pAD1 of *E. faecalis*.**
traC	**Hemolytic bacteriocin from pAD1 of *E. faecalis*.**
pneu	**Pneumolysin from *S. pneumonae*.**
sly	**Cytolytic toxin from *Streptococcus suis*.**
slo	**Streptolysin O from plasmid pMK157 *Streptococcus cannis*.**
slo	**Streptolysin O from group A, C and G *Streptococci*.**
sagC	**Streptolysin S**
L50	**Enterocin L50 from *E. faecium*.**
aph	**Resistance to aminoglycoside antibiotics (gentamycin, kanamycin) from *E. casseliflavus*.**
genta	**Newly characterized resistance gene to gentamycin from *S. aureus*.**
ermAM	**Resistance to erythromycin from plasmid pAM-b-1 of *S. faecalis*.**
ery	**Resistance to erythromycin from transposon Tn917.**
penA	**Class AmpC b-lactamase from *S. pneumoniae* giving resistance to penicillin antibiotics.**
orf14	**Orf14 protein of transposon Tn916 of *E. faecalis*.**
orf1	**Orf1 protein of insertion sequence (mobile DNA element) IS232 of *B. thuringiensis*.**
tetM	**Tetracycline Resistance from transposon Tn916 of *E. faecalis*.**
esp	**Surface protein of virulent *Enteroccoci* clinical isolates.**

administered until successful elimination of the pathogenic Enterococcus faecium is achieved.

As used in the present application, the term "substantially reduce" indicates that the number of bacteria is reduced to a number which can be completely eliminated by the animal's defense system or by using conventional antibacterial therapies.

The present invention will be particularly useful in treating critically ill patients or those with severe underlying disease or immunosuppression (e.g. patients in ICUs or in oncology or transplant wards), patients who have had an intraabdominal or cardio-thoracic surgical procedure or an indwelling urinary or central venous catheter, and persons who have had a prolonged hospital stay or received multi-antimicrobial and/or vancomycin therapy.

Deposits of ENB6 (ATCC # PTA-40) and ENB13 (ATCC # PTA-39) were made on May 12, 1999 at the American Type Culture Collection, 10801 University Blvd., Manassas, VA. 20110-2209.

The foregoing embodiments of the present invention are further described in the following Examples. However, the present invention is not limited by the Examples, and variations will be apparent to those skilled in the art.

EXAMPLES

1. VREF Bacteremia Rescue Experiment #1: Dose-Finding Study

Figures 3 and 4 show the results of a dose-finding study.

Materials and Methods:

We had previously determined that the $2xLD_{50}$ dose for a clinical VREF isolate designated CRMEN44 is 1×10^9 CFU, when injected I.P. into one month-old balb/c female mice. In other studies (data not shown here), we had determined that the I.P. injection of this bacteria strain causes a bacteremia within 15 minutes, and that the I.P. injection of phage ENB6 causes a viremia within 15 minutes. In this study, the following dosages of phage ENB6 were administered once (and only once) I.P., exactly ½ hour after the bacterial challenge: 3×10^9, 3×10^8, 3×10^6, and 3×10^4 PFU plaque forming units (PFU). In addition, a dose of 3×10^9 PFU was administered I.P. to another set of animals, as a control, with no bacterial challenge.

The non-parametric rating scale for observable signs of illness is as follows: 5 = Normal animal; 4 = Mild lethargy; 3 = Mild lethargy + Ruffled fur; 2 = the above, plus exudate around the eyes; 1 = Moribund; and 0 = Dead.

Results:

Phage administered as a control did not produce any detectable symptoms in the animals. Bacteria administered without any phage treatment caused the death of all the animals, within 48 hours. With the two highest dosages of phage there were

no deaths, and the animals recovered within 24 hours from the minimal signs of illness that had developed, with no relapse over a period of 21 days of observation. While there were some deaths with the two lowest dosages of phage, nevertheless roughly half the animals in these groups survived (and recovered completely) after becoming moderately ill.

Discussion:

Phage ENB6 rescues animals from an otherwise-fatal dose of VREF, a bacterial pathogen for which no consistently reliable antibiotic is currently available. The infection here is fulminant, using a concentration of bacteria (10^9, which will be very concentrated in the 3 ml of blood in a mouse's circulatory system) that is orders-of-magnitude greater than that found in bacteremic humans (where titers in blood reach only 10^2 to 10^4 CFU per cc).

Conclusion:

While an IND approval will be required from the FDA before such phages can be administered therapeutically to humans, phage strains that lyse VREF hosts *in-vitro* should be able to kill the bacterial targets wherever encountered (*in-vivo*), whether within the mouse or the human circulatory system. Moreover, multiple phage doses will be employed in treating humans. In this experiment only one dose was administered, in order to demonstrate the ability of the phages to grow exponentially in number and to thereby overwhelm the target bacteria.

2. VREF Bacteremia Rescue Experiment #2: Delayed Treatment

Figures 5 and 6 show the results of delay in the treatment of a fulminant bacteremia.

Materials and Methods:

Same as in Experiment 1, except for the dosage and timing of the phage administration. In this experiment, only the highest dose (3×10^9) of phage ENB6 was administered. After the I.P. bacterial challenge, the one (and only one) I.P. administration of the phage dose was delayed until one or another of the following time points: 2, 5, 8, 14, 18 and 24 hours. One group of animals received no phage treatment, as a control.

Results:

With no treatment, all animals were dead within 48 hours. With treatment delayed 2 hours and 5 hours, all animals survived (after becoming moderately ill). With treatment delayed from 8 – 24 hours approximately half the animals died, but for the half that survived, even though the degree of illness reached was severe, nevertheless there was full and complete recovery by day 4 or 5, with no relapse.

Discussion:

Even when treatment of a fulminant bacteremia in mice is delayed, phage ENB6 tends to rescue the animals from an otherwise-fatal dose of VREF. The rescue is

100% with delays up to and including 5 hours. With delays between 8 – 24 hours, approximately 50% of the animals survive and go on to recover completely.

Conclusion:

While an IND approval will be required from the FDA before such phages can be administered therapeutically to humans, phage strains that lyse VREF hosts *in-vitro* should be able to kill the bacterial targets wherever encountered (*in-vivo*), whether within the mouse or the human circulatory system. In the human, concentrations of VREF are orders-of-magnitude lower than the concentrations achieved here, so it should be that much easier to achieve a therapeutic success. Moreover, in treating humans, multiple administration of phage will be employed. In this experiment only one dose was administered, in order to demonstrate the ability of the phages to grow exponentially in number and to thereby overwhelm the target bacteria.

We claim:

1. A wild-type phage which is lytic for strains of vancomycin-resistant *Enterococcus faecium* (VREF) as well as for strains of vancomycin-sensitive *Enterococcus faecium* (VSEF), wherein said phage is selected from the group consisting of ENB6 (ATCC# PTA-40), and ENB13 (ATCC# PTA-39)

2. A method for treating an *Enterococcus faecium* infection comprising administering an amount of a phage selected from the group consisting of ENB6 (ATCC# PTA-40), and ENB13 (ATCC# PTA-39) effective to eradicate or substantially reduce an *Enterococcus faecium* infection to a patient in need of such treatment.

3. The method according to claim 2, wherein said *Enterococcus faecium* is vancomycin-resistant *Enterococcus faecium*.

4. The method according to claim 2, wherein said phage is administered by a route selected from the group consisting of orally, topically, intravenously, intra-arterially, intraperitoneally, intrathecally, by inhalation, by nasal spray, by irrigation of a wound, by suppository, and by enema.

5. The method according to claim 2, wherein said phage is administered at a total dose of between $10^3 - 10^{12}$ PFU.

6. The method according to claim 5, wherein said phage is administered at a total dose of between $10^5 - 10^{11}$ PFU.

7. The method according to claim 2, further comprising administering an antibiotic.

8. A method for reducing the probability of an *Enterococcus faecium* colonization becoming an infection comprising administering an amount of phage selected from the group consisting of ENB6 (ATCC# PTA-40), and ENB13 (ATCC#-PTA-39) effective to reduce the probability of such colonization becoming an infection to a patient at risk for an *Enterococcus faecium* infection.

9. The method according to claim 8, wherein said phage is administered by a route selected from the group consisting of orally, topically, intravenously, intra-arterially, intraperitoneally, intrathecally, by inhalation, by nasal spray, by irrigation of a wound, by suppository, and by enema.

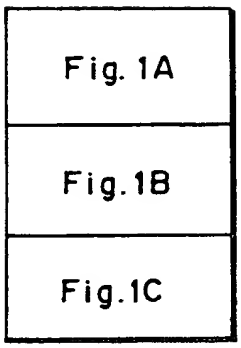
10. The method according to claim 8, wherein said phage is administered at a total dose of between $10^3 - 10^{12}$ PFU.

11. The method according to claim 10, wherein said phage is administered at a total dose of between $10^5 - 10^{11}$ PFU.

12. A pharmaceutical composition comprising a phage selected from the group consisting of ENB6 (ATCC# PTA-40), and ENB13 (ATCC# PTA-39) in combination with a pharmaceutical carrier.

13. The composition according to claim 12, further comprising an antibiotic.

Fig. 1



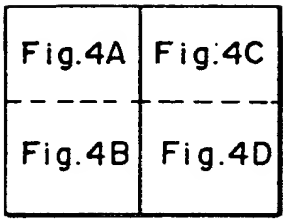


Fig. 4

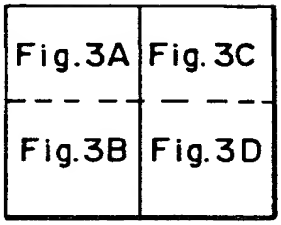


Fig. 3

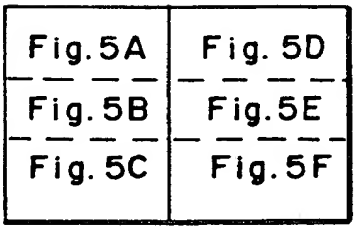


Fig. 5

Legend

- Phage ($3x10^9$pfu) only
- VRE (10^9cfu) + Phage ($3x10^9$pfu)
- VRE (10^9cfu) + Phage ($3x10^8$pfu)
- VRE (10^9cfu) + Phage ($3x10^6$pfu)
- VRE (10^9cfu) + Phage ($3x10^4$pfu)
- VRE(10^9cfu) only

Symptoms of Disease

0 => Death
1 => Moribund
2 => Ruffled fur, hunchback & closed eyes
3 => Ruffled fur & hunchback
4 => Decreased activity
5 => Normal mouse

Fig. 1A

Alignments of hypothetical amino acid sequences predicted from ENB6 DNA with the protein databases.

BLASTX alignments of the highest score (Expect Value less than 0.005) to proteins of potentially undesirable factors are shown. "Query" = putative protein from ENB6; "Sbjct" = target protein found in database. Numbers = codon or amino acid number; {} = total length of target protein.

1. Plasminogen-binding protein MLC36 from group C Streptococcus sp.

```
Query: 70   RTWTEYLATGHVHDKNHAKQLERLSKRDISLGDVATVVDFMSRRNDGYITALIEQNSVNE
249
            R  T     T +V +K  A +LE+L    +++  D  ++VD M   ND  T   +   + +
Sbjct: 36   RLVTNMWKTQYVKEKQRADELEKLLHSEVA-DYNSLVDKMKVVNDSLQTTKRDYEEIEK
93

Query: 250  KLFNKL----GVTDKMRNEAKA---------EYEVELKQAQEEIKKLQEELAEKLQKGE*Y
393
            +L NKL      + +K++N+  +           E +++L Q    +  L+ EL ++ QK E
Sbjct: 94   ELGNKLKENQDLEEKLKNKEFSLGEALRYINELDLKLGQLNIDNIDLKHELEQEKQKAEAY
154 {454}
```

Fig. 1B

2. IstA protein homolog from *Bacillus thuringiensis* similar to ORF1 protein of insertion sequence IS232

```
Query: 871 QFAYDFAFSGYPQLAGMPPSSGQVDAPQMI 960
           QFA DF F    P +AG P + G+V+AP  +
Sbjct: 239 QFAQDFGFKVQPCIAGRPNTKGKVEAPMKL 268 (431)
```

3. Hemagglutinin protein from Influenza A virus

```
Query: 2636 NQAVLARNREFNKIQREGAYLDHLIEGLKEHLSEE--------------LENTNTLKYIE
2499
              N+ +   N +F++I++E + ++  I+ L++++ +                 LEN NT+   +
Sbjct: 398  NRVIEKTNEKFHQIEKEFSEVEGRIQDLEKYVEDTKIDLWSYNAELLVALENQNTIDLTD
457

Query: 2498 PELRVKGKPSDREMILCLSDWHIGAF----------VNNIDTGGYNYDIFR-ERLNS
2361
               E+    + + R++     D   G F          + +I  G YN+DI+R E LN+
Sbjct: 458  SEMNKLFEKTRRQLRENAEDMGNGCFKIYHKCDNACIESIRNGTYNHDIYRDEALNN
514 (566)
```

Fig. 1C

4. *Bacillus subtilis* protein similar to ORF14 of *E. faecalis* transposon TN916

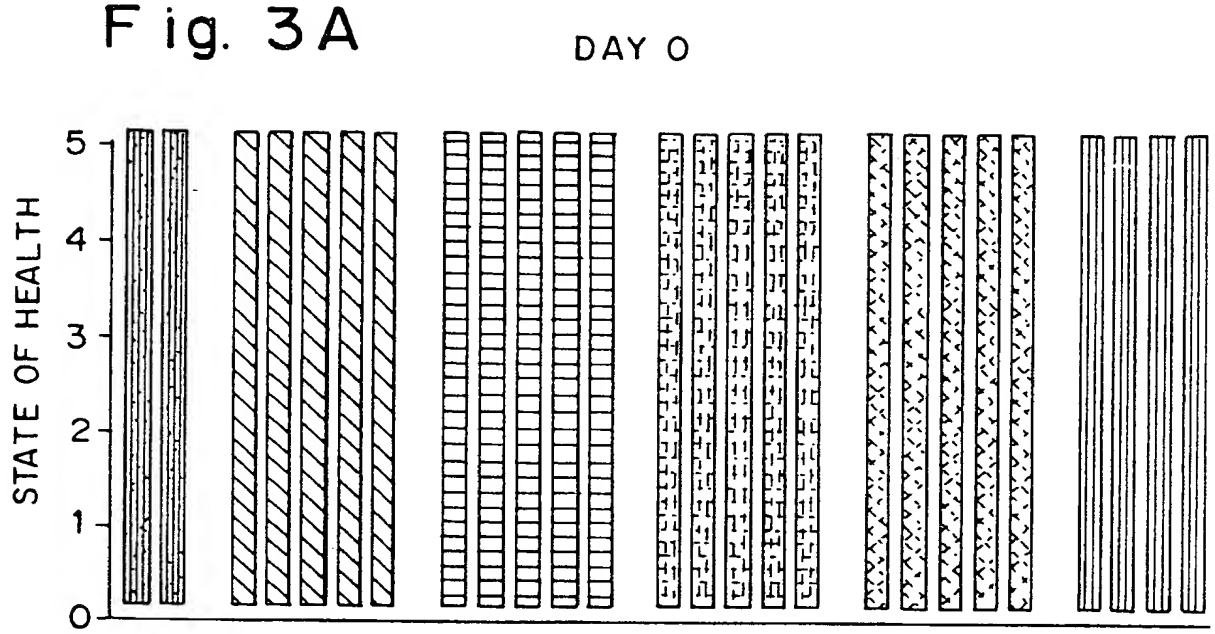
```
Query: 2523 YDWGGGRTGRDPFESSPIATDCSSFVWWCFKHAGVELNGGATGMTTWSIIADTKLETIAT
2344
            Y WGG      +          DCS  V W F  AG+ L   A
Sbjct: 224  YAWGGS--------------NPETGFDCSGLVQWSFAKAGITLPRTAQEQ----------
259

Query: 2343 RGQKNSAIFDKMKAGDIIWF------RNCEHIGIYCGEGKMVACNGSGNMNESPTAGIIV
2182
             G        +  AGD+++F      +   H+GIY G G+M   N SG          I
Sbjct: 260  HGATKKISEKEATAGDLVFFGGTYEGKAITHVGIYVGNGRMFNSNDSG---------IQY
310

Query: 2181 SDMTSGYWWD  2152
            SD+ SGYW D
Sbjct: 311  SDLKSGYWRD   320  {329}
```

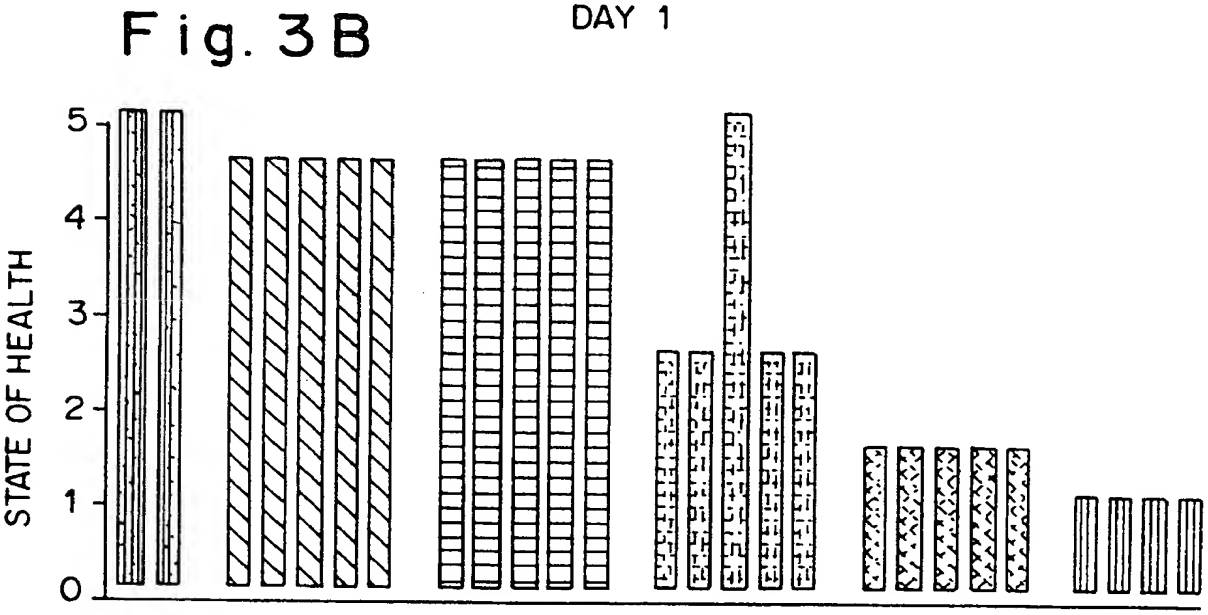
Fig. 2

Fig. 3A



EFFECT OF PHAGE CONCENTRATION ON VRE INFECTED MICE

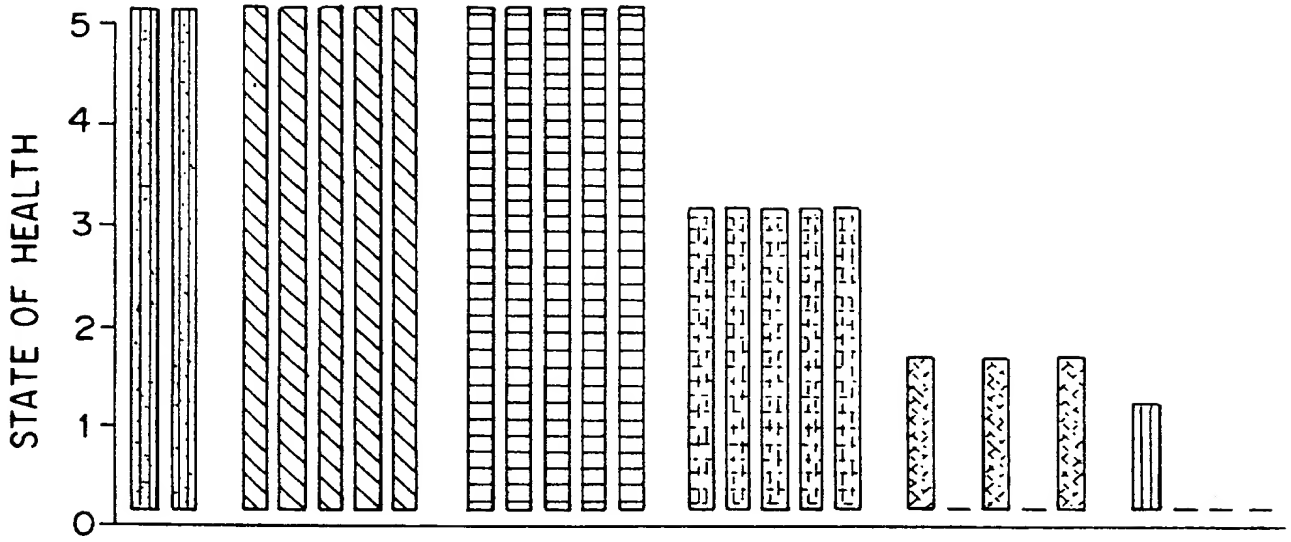
Fig. 3B



EFFECT OF PHAGE CONCENTRATION ON VRE INFECTED MICE

Fig. 3C

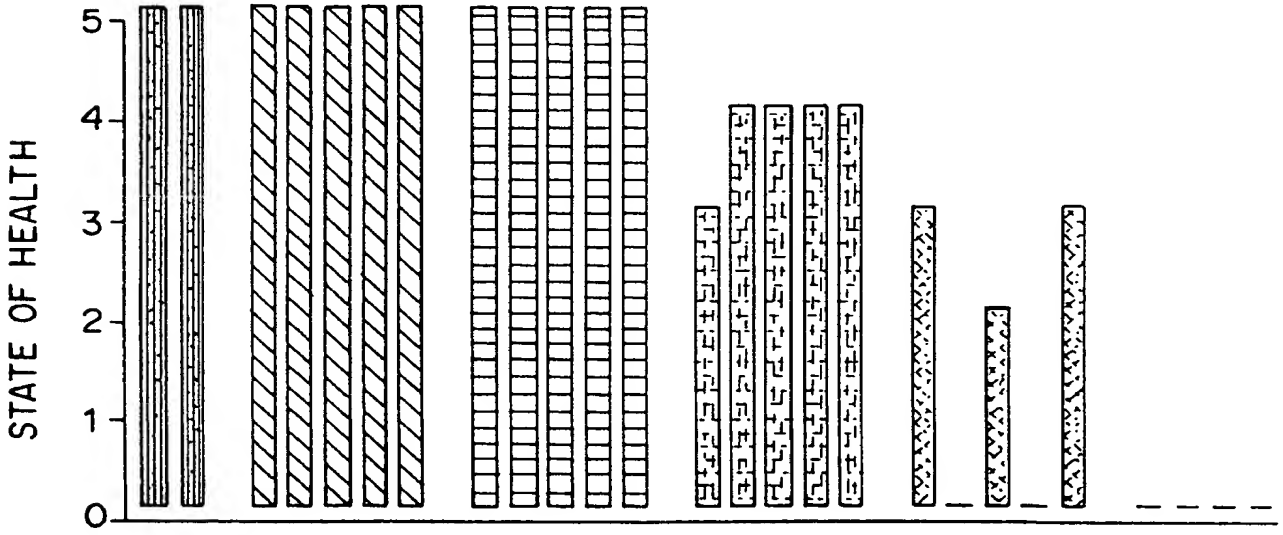
DAY 2



EFFECT OF PHAGE CONCENTRATION ON VRE INFECTED MICE

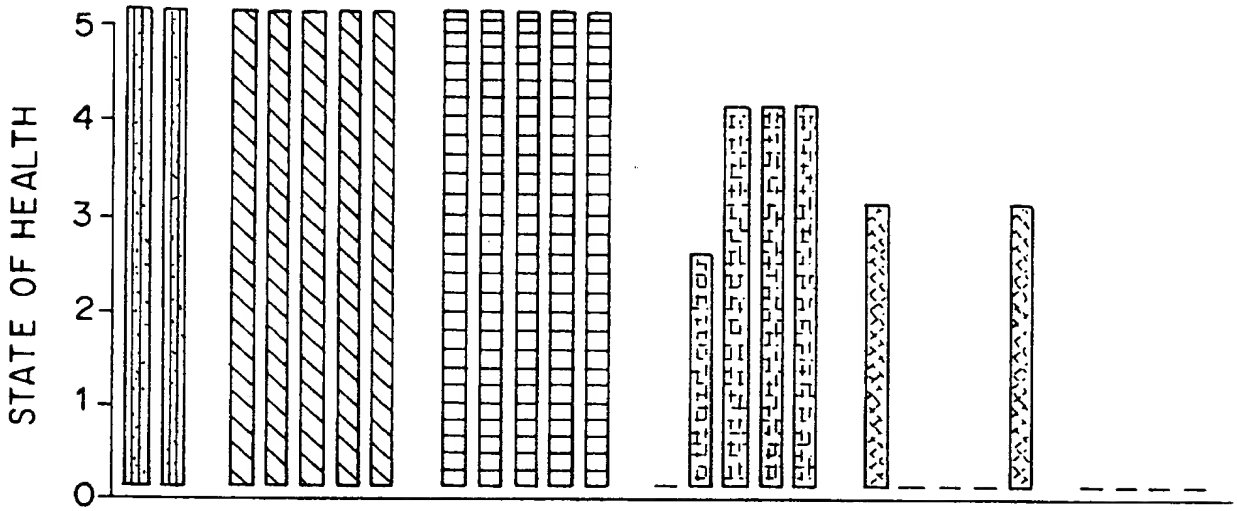
Fig. 3D

DAY 3



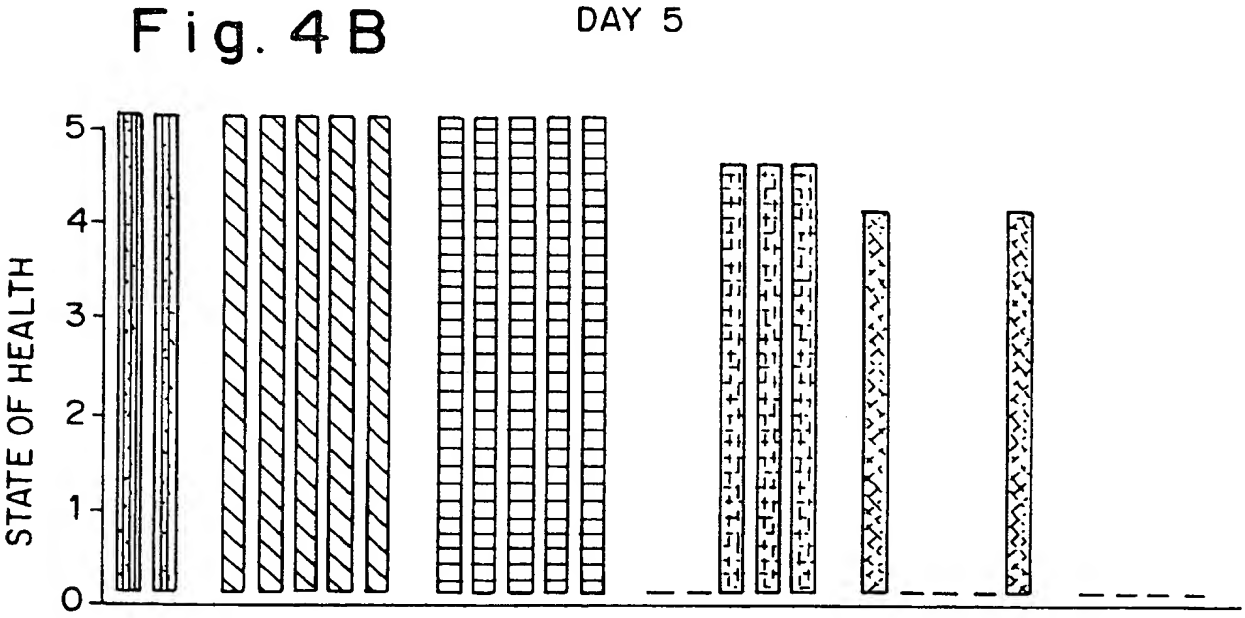
EFFECT OF PHAGE CONCENTRATION ON VRE INFECTED MICE

Fig. 4A



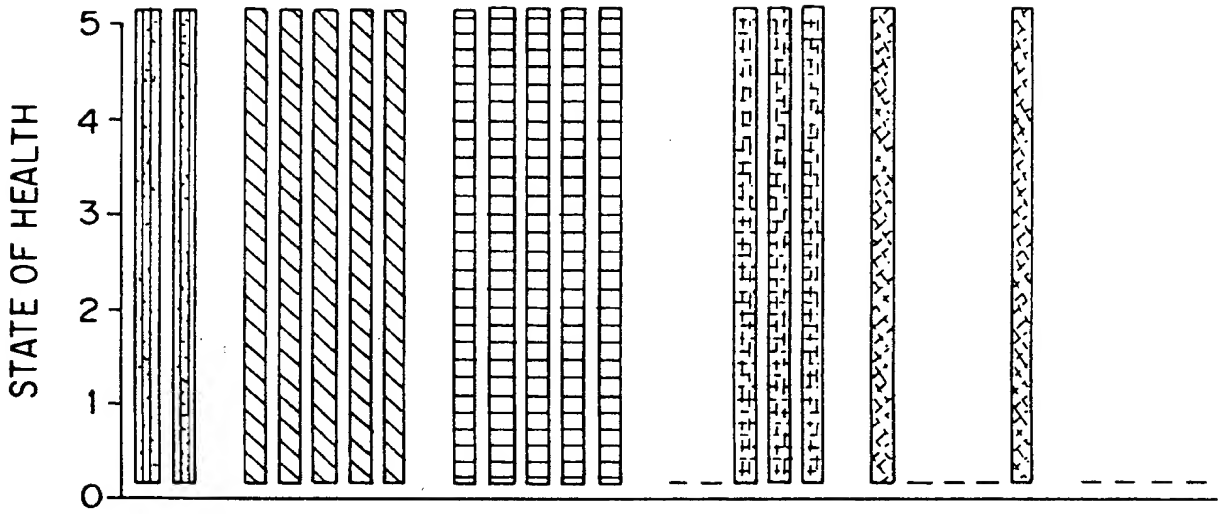
EFFECT OF PHAGE CONCENTRATION ON VRE INFECTED MICE

Fig. 4B



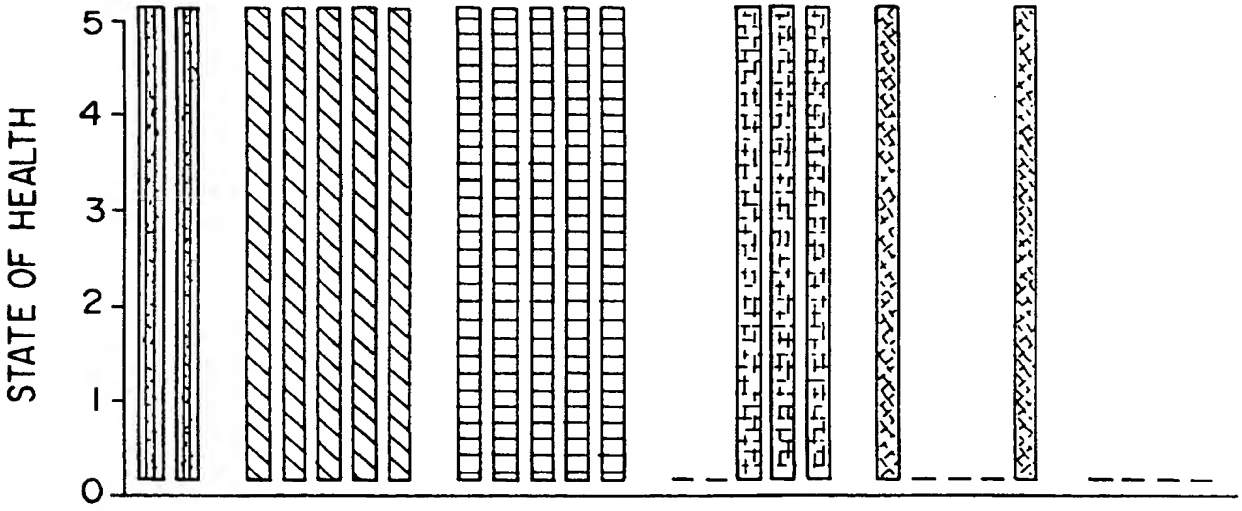
EFFECT OF PHAGE CONCENTRATION ON VRE INFECTED MICE

Fig. 4C DAY 6



EFFECT OF PHAGE CONCENTRATION ON VRE INFECTED MICE

Fig. 4D DAY 20



EFFECT OF PHAGE CONCENTRATION ON VRE INFECTED MICE

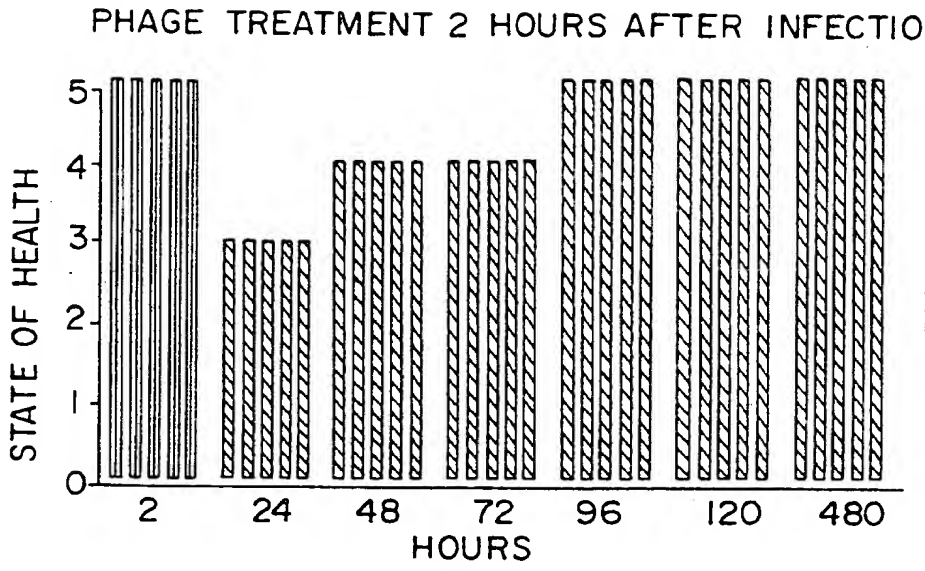


Fig.5A

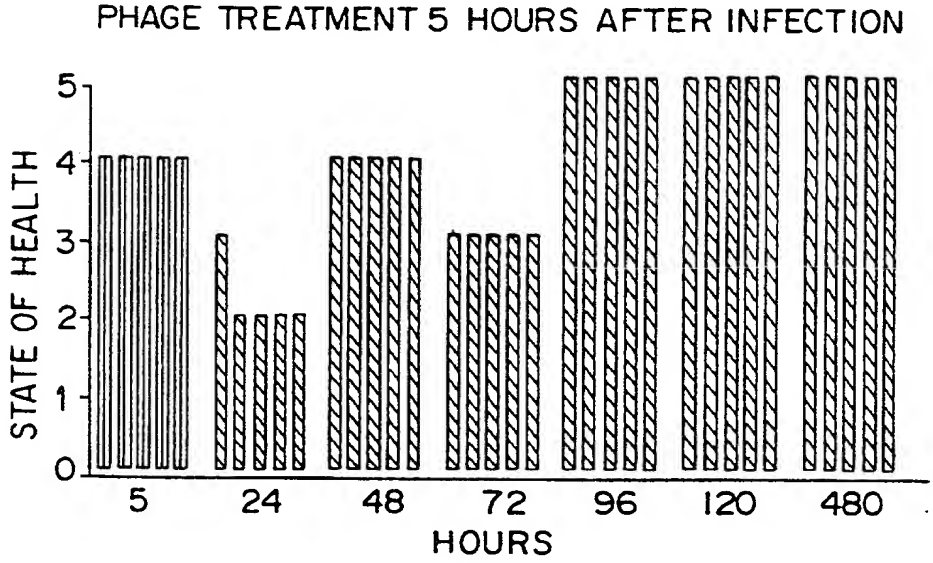


Fig.5B

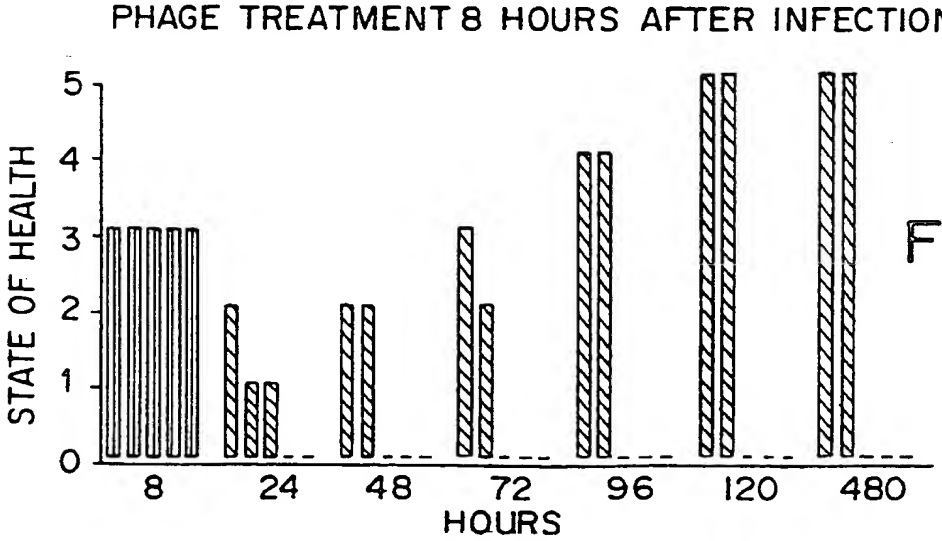


Fig.5C

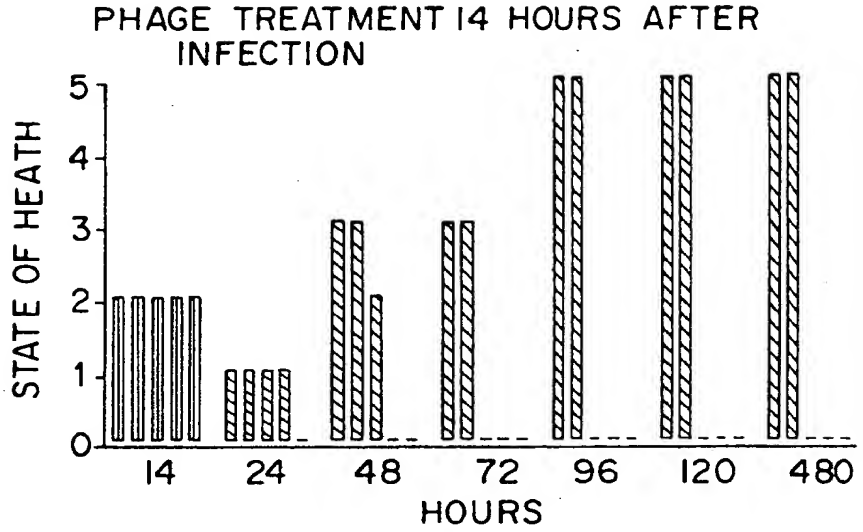


Fig. 5D

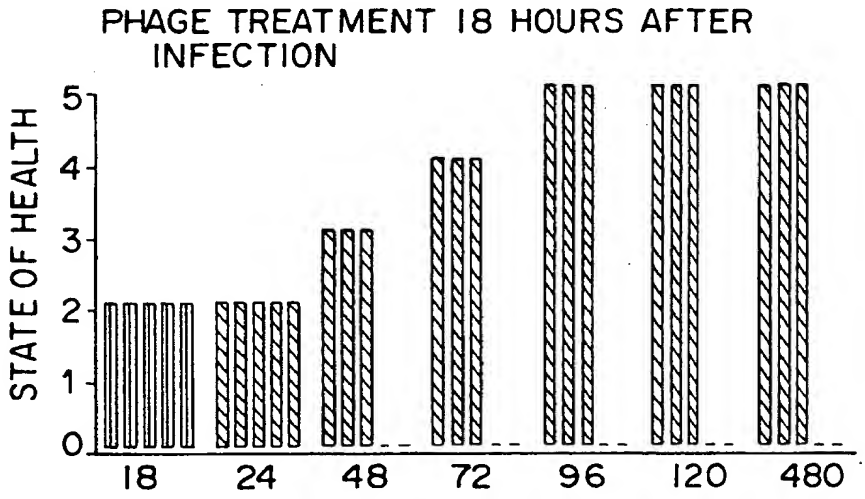


Fig. 5E

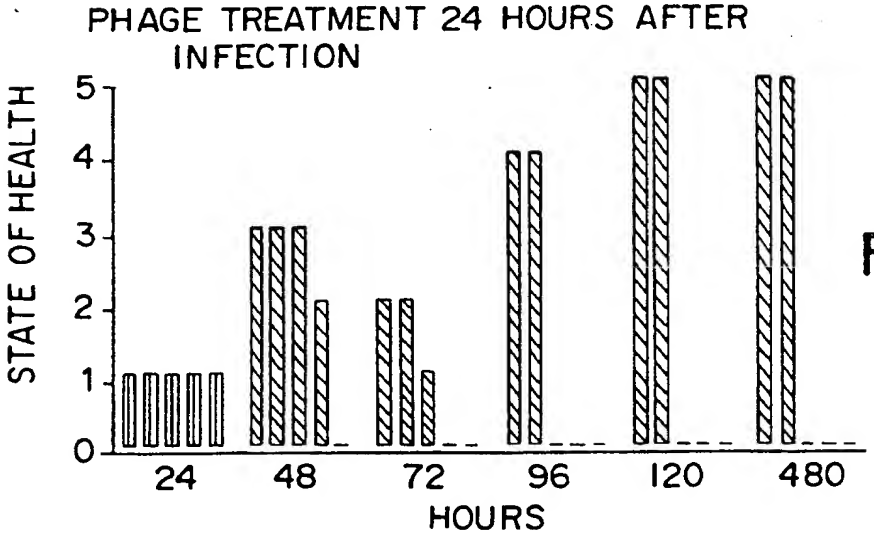


Fig. 5F

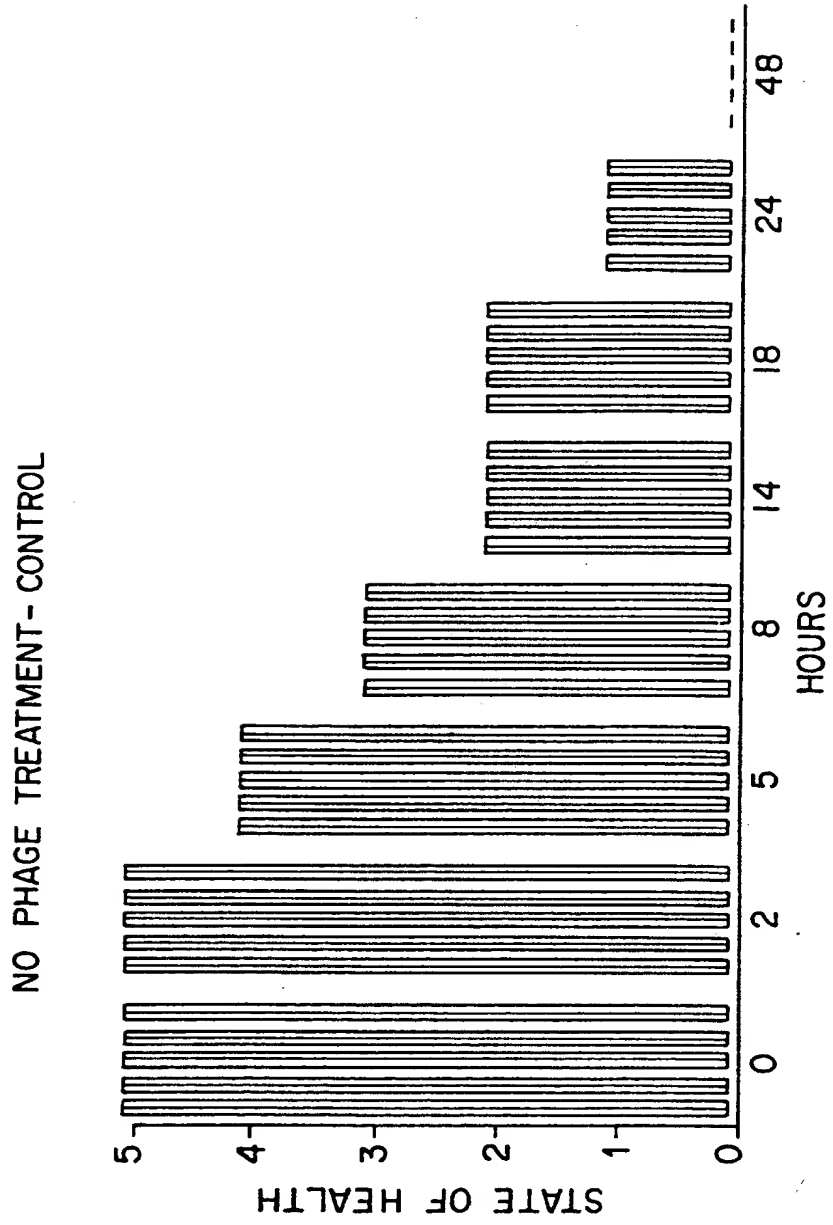

NO PHAGE TREATMENT - CONTROL
STATE OF HEALTH
0
1
2
3
4
5
0
2
5
8
14
18
24
48
HOURS

Fig. 6

PATENT COOPERATION TREATY

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To: ROBERT B. MURRAY
ARENT FOX KINTNER PLOTKIN & KAHN, PLLC
1050 CONNECTICUT AVENUE, N.W.
SUITE 600
WASHINGTON, DC 20036-5339

PCT

WRITTEN OPINION

(PCT Rule 66)

Date of Mailing *(day/month/year)*	29 MAY 2001
Applicant's or agent's file reference: F108026-00001	REPLY DUE within ONE months from the above date of mailing

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/US00/06718	12 MAY 2000	12 MAY 1999

International Patent Classification (IPC) or both national classification and IPC
IPC(7): A01N 63/00; A61K 39/02 and US Cl.: 424/93.6, 234.1

Applicant
EXPONENTIAL BIOTHERAPIES, INC.

1. This written opinion is the first (first, etc.) drawn by this International Preliminary Examining Authority.

2. This opinion contains indications relating to the following items:

- I [X] Basis of the opinion
- II [ ] Priority
- III [ ] Non-establishment of opinion with regard to novelty, inventive step or industrial applicability
- IV [ ] Lack of unity of invention
- V [X] Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI [ ] Certain documents cited
- VII [ ] Certain defects in the international application
- VIII [X] Certain observations on the international application

3. The applicant is hereby invited to reply to this opinion.

When? See the time limit indicated above. ~~The applicant may, before the expiration of that time limit, request this Authority to grant an extension., see Rule 66.2(d).~~

How? By submitting a written reply, accompanied, where appropriate, by amendments, according to Rule 66.3. For the form and the language of the amendments, see Rules 66.8 and 66.9.

Also For an additional opportunity to submit amendments, see Rule 66.4.
For the examiner's obligation to consider amendments and/or arguments, see Rule 66.4 *bis*.
For an informal communication with the examiner, see Rule 66.6.

If no reply is filed, the international preliminary examination report will be established on the basis of this opinion.

4. The final date by which the international preliminary examination report must be established according to Rule 69.2 is: 12 SEPTEMBER 2001

Name and mailing address of the IPEA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Shanon A. Foley TERRY J. DEY PARALEGAL SPECIALIST TECHNOLOGY CENTER 1600
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

Form PCT/IPEA/408 (cover sheet) (July 1998) *

WRITTEN OPINION

International application No.
PCT/US00/06718

I. Basis of the opinion

1. With regard to the **elements** of the international application: *

[X] the international application as originally filed

[X] the description:
pages 1-13 , as originally filed
pages NONE , filed with the demand
pages NONE , filed with the letter of

[X] the claims:
pages 14-16 , as originally filed
pages NONE , as amended (together with any statement) under Article 19
pages NONE , filed with the demand
pages NONE , filed with the letter of

[X] the drawings:
pages 1-12 , as originally filed
pages NONE , filed with the demand
pages NONE , filed with the letter of

[X] the sequence listing part of the description:
pages NONE , as originally filed
pages NONE , filed with the demand
pages NONE , filed with the letter of

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.
These elements were available or furnished to this Authority in the following language _____ which is:

[ ] the language of a translation furnished for the purposes of international search (under Rule 23.1(b)).

[ ] the language of publication of the international application (under Rule 48.3(b)).

[ ] the language of the translation furnished for the purposes of international preliminary examination (under Rules 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the written opinion was drawn on the basis of the sequence listing:

[ ] contained in the international application in printed form.

[ ] filed together with the international application in computer readable form.

[ ] furnished subsequently to this Authority in written form.

[ ] furnished subsequently to this Authority in computer readable form.

[ ] The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

[ ] The statement that the information recorded in computer readable form is identical to the writen sequence listing has been furnished.

4. [X] The amendments have resulted in the cancellation of:

[X] the description, pages none

[X] the claims, Nos. none

[X] the drawings, sheets/~~fig~~ none

5. [ ] This opinion has been drawn as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).

** Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this opinion as "originally filed".*

V. Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. statement

Novelty (N)	Claims	1-13	YES
	Claims	NONE	NO
Inventive Step (IS)	Claims	1-13	YES
	Claims	NONE	NO
Industrial Applicability (IA)	Claims	1-13	YES
	Claims	NONE	NO

2. citations and explanations

Claims 1-13 meet the criteria set out in PCT Article 33(2)-(4), because the prior art does not teach or fairly suggest treating vancomycin-sensitive *Enterococcus faecium* with phage.

----------NEW CITATIONS----------

BRANDT et al. Effective Treatment of Multidrug-Resistant Enterococcal Experimental Endocarditis with Combinations of Cell Wall-Active Agents. The Journal of Infectious Diseases. 1996. Vol. 173, pages 909-913, see the abstract.

SMITH et al. The Control of Experimental Escherichia coli Diarrhoea in Calves by Means of Bacteriophages. The Journal of Microbiology. 1997. Vol. 133, pages 1111-1126, see the abstract.

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

Claims 1-13 are objected to as lacking clarity under PCT Rule 66.2(a)(v) because practice of the claimed invention is not enabled as required under PCT Rule 5.1(a) for the reasons set forth in the immediately preceding paragraph.

The description is objected to under PCT Rule 66.2(a)(v) as lacking clarity under PCT Article 5 because it fails to adequately enable practice of the claimed invention because: The claims are not commensurate with the scope of the disclosure. The discosure is directed to using a particular bacteriophage, ENB6, to reduce illness caused by a specific vancomycin resistant *Enterococcus faecium* (VREF) design... CREMEN44, see page 10, line 5 through page 11, line 12 and page 12, line 5 through page 13, line 2. The disclosure only offers only speculation as to the *in vivo* effectiveness other phage strains would have on other VREF hosts from assays conducted *in vitro*. Merril et al. discloses a method of treating drug resistant bacteria such as Enterococcus, by administering 10^6 to 10^{12} pfu/kg/day of bacteriophages, see claims 1, 2, 4, 6, 9, and 13, but does not teach the particular strain of Enterococcus claimed. Brandt et al. teaches the only known effective treatment against a vancomycin resistent strain of Enterococcus faecium with several antibiotics, see the abstract. Smith et al. teaches several phages used in the treatment of enteropathic strains of *E. coli* and teaches that only a particular phage was effective at controlling a particular strain of *E. coli*, see the abstract. Based on the teachings of the references, one skilled in the art would doubt the efficacy of administering a particular baceriophage against all strains of vancomycin-sensitive *Enterococcus faecium*.

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

To:

MURRAY, Robert, B.
Arent Fox Kintner Plolkin & Kahn,
PLLC
Suite 600
1050 Connecticut Avenue, N.W.
Washington, DC 20036-5339
ETATS-UNIS D'AMERIQUE

INFORMATION CONCERNING ELECTED OFFICES NOTIFIED OF THEIR ELECTION

(PCT Rule 61.3)

Date of mailing (day/month/year) 26 March 2001 (26.03.01)	
Applicant's or agent's file reference F108026-0000 /	**IMPORTANT INFORMATION**

International application No.	**International filing date** (day/month/year)	**Priority date** (day/month/year)
PCT/US00/06718	12 May 2000 (12.05.00)	13 May 1999 (13.05.99)

Applicant
EXPONENTIAL BIOTHERAPIES, INC. et al

1. The applicant is hereby informed that the International Bureau has, according to Article 31(7), notified each of the following Offices of its election:

   AP :GH,GM,KE,LS,MW,SD,SL,SZ,TZ,UG,ZW
   EP :AT,BE,CH,CY,DE,DK,ES,FI,FR,GB,GR,IE,IT,LU,MC,NL,PT,SE
   National :AU,BG,CA,CN,CZ,DE,IL,JP,KP,KR,MN,NO,NZ,PL,RO,RU,SE,SK,US

2. The following Offices have waived the requirement for the notification of their election; the notification will be sent to them by the International Bureau only upon their request:

   EA :AM,AZ,BY,KG,KZ,MD,RU,TJ,TM
   OA :BF,BJ,CF,CG,CI,CM,GA,GN,GW,ML,MR,NE,SN,TD,TG
   National :AE,AG,AL,AM,AT,AZ,BA,BB,BR,BY,CH,CR,CU,DK,DM,DZ,EE,ES,FI,GB,GD,
   GE,GH,GM,HR,HU,ID,IN,IS,KE,KG,KZ,LC,LK,LR,LS,LT,LU,LV,MA,MD,MG,MK,MW,MX,
   PT,SD,SG,SI,SL,TJ,TM,TR,TT,TZ,UA,UG,UZ,VN,YU,ZA,ZW

3. The applicant is reminded that he must enter the "national phase" **before the expiration of 30 months from the priority date** before each of the Offices listed above. This must be done by paying the national fee(s) and furnishing , if prescribed, a translation of the international application (Article 39(1)(a)), as well as, where applicable, by furnishing a translation of any annexes of the international preliminary examination report (Article 36(3)(b) and Rule 74.1).

   Some offices have fixed time limits expiring later than the above-mentioned time limit. For detailed information about the applicable time limits and the acts to be performed upon entry into the national phase before a particular Office, see Volume II of the PCT Applicant's Guide.

   The entry into the European regional phase is postponed **until 31 months from the priority date** for all States designated for the purposes of obtaining a European patent.

The International Bur au f WIPO
34, chemin des Colombettes
1211 Gen va 20, Switzerland

Facsimile No. (41-22) 740.14.35

Authorized officer:
Claudio Borton

Telephone No. (41-22) 338.83.38

PATENT COOPERATION TREATY

PCT

From the INTERNATIONAL BUREAU

To:
MURRAY, Robert, B.
Arent Fox Kintner Plotkin & Kahn,
PLLC
Suite 600
1050 Connecticut Avenue, N.W.
Washington, DC 20036-5339
ETATS-UNIS D'AMERIQUE

NOTICE INFORMING THE APPLICANT OF THE COMMUNICATION OF THE INTERNATIONAL APPLICATION TO THE DESIGNATED OFFICES

(PCT Rule 47.1(c), first sentence)

Date of mailing (day/month/year) 23 November 2000 (23.11.00)	
Applicant's or agent's file reference F108026-0000 1	**IMPORTANT NOTICE**

International application No.	**International filing date** (day/month/year)	**Priority date** (day/month/year)
PCT/US00/06718	12 May 2000 (12.05.00)	13 May 1999 (13.05.99)

Applicant
EXPONENTIAL BIOTHERAPIES, INC. et al

1. Notice is hereby given that the International Bureau has communicated, as provided in Article 20, the international application to the following designated Offices on the date indicated above as the date of mailing of this Notice:
   AG,AU,DZ,KP,KR,US

   In accordance with Rule 47.1(c), third sentence, those Offices will accept the present Notice as conclusive evidence that the communication of the international application has duly taken place on the date of mailing indicated above and no copy of the international application is required to be furnished by the applicant to the designated Office(s).

2. The following designated Offices have waived the requirement for such a communication at this time:
   AE,AL,AM,AP,AT,AZ,BA,BB,BG,BR,BY,CA,CH,CN,CR,CU,CZ,DE,DK,DM,EA,EE,EP,ES,FI,GB,GD,
   GE,GH,GM,HR,HU,ID,IL,IN,IS,JP,KE,KG,KZ,LC,LK,LR,LS,LT,LU,LV,MA,MD,MG,MK,MN,MW,MX,
   NO,NZ,OA,PL,PT,RO,RU,SD,SE,SG,SI,SK,SL,TJ,TM,TR,TT,TZ,UA,UG,UZ,VN,YU,ZA,ZW
   The communication will be made to those Offices only upon their request. Furthermore, those Offices do not require the applicant to furnish a copy of the international application (Rule 49.1(a-bis)).

3. Enclosed with this Notice is a copy of the international application as published by the International Bureau on
   23 November 2000 (23.11.00) under No. WO 00/69269

REMINDER REGARDING CHAPTER II (Article 31(2)(a) and Rule 54.2)

If the applicant wishes to postpone entry into the national phase until 30 months (or later in some Offices) from the priority date, **a demand for international preliminary examination** must be filed with the competent International Preliminary Examining Authority before the expiration of 19 months from the priority date.

It is the applicant's sole responsibility to monitor the 19-month time limit.

Note that only an applicant who is a national or resident of a PCT Contracting State which is bound by Chapter II has the right to file a demand for international preliminary examination.

REMINDER REGARDING ENTRY INTO THE NATIONAL PHASE (Article 22 or 39(1))

If the applicant wishes to proceed with the international application in the **national phase**, he must, within 20 months or 30 months, or later in some Offices, perform the acts referred to therein before each designated or elected Office.

For further important information on the time limits and acts to be performed for entering the national phase, see the Annex to Form PCT/IB/301 (Notification of Receipt of Record Copy) and Volume II of the PCT Applicant's Guide.

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No. (41-22) 740.14.35

Authorized officer
J. Zahra

Telephone No. (41-22) 338.83.38

+We claim:

1. A wild-type phage which is lytic for susceptible strains of vancomycin-resistant *Enterococcus faecium* (VREF) as well as for susceptible strains of vancomycin-sensitive *Enterococcus faecium* (VSEF), wherein said phage is selected from the group consisting of ENB6 (ATCC# PTA-40), and ENB13 (ATCC# PTA-39)

2. A method for treating an *Enterococcus faecium* infection comprising administering an amount of a phage selected from the group consisting of ENB6 (ATCC# PTA-40), and ENB13 (ATCC# PTA-39) effective to eradicate or substantially reduce an *Enterococcus faecium* infection to a patient in need of such treatment, and lysing a susceptible strain of *Enterococcus faecium* causing said infection with said phage.

3. The method according to claim 2, wherein said Enterococcus faecium is vancomycin-resistant *Enterococcus faecium*.

4. The method according to claim 2, wherein said phage is administered by a route selected from the group consisting of orally, topically, intravenously, intra-arterially, intraperitoneally, intrathecally, by inhalation, by nasal spray, by irrigation of a wound, by suppository, and by enema.

5. The method according to claim 2, wherein said phage is administered at a total dose of between 10^5 - 10^{12} pfu/kg/day.

6. The method according to claim 5, wherein said phage is administered at a total dose of between 10^5 - 10^{11} pfu/kg/day.

7. The method according to claim 2, further comprising administering an antibiotic.

8. A method for reducing the probability of an *Enterococcus faecium* colonization becoming an infection comprising administering an amount of phage selected from the group consisting of ENB6 (ATCC# PTA-40), and ENB13 (ATCC# PTA-39) effective to reduce the probability of such colonization becoming an infection to a patent at risk for an *Enterococcus faecium* infection, and lysing a susceptible strain of *Enterococcus faecium* comprising said colonization with said phage.

9. The method according to claim 8, wherein said phage is administered by a route selected from the group consisting of orally, topically, intravenously, intra-arterially, intraperitoneally, intrathecally, by inhalation, by nasal spray, by irrigation of a wound, by suppository, and by enema.

10. The method according to claim 8, wherein said phage is administered at a total dose of between 10^3 - 10^{12} pfu/kg/day.

11. The method according to claim 10, wherein said phage is administered at a total dose of between 10^5 - 10^{11} pfu/kg/day.

12. A pharmaceutical composition comprising a phage selected from the group consisting of ENB6 (ATCC# PTA-40), and ENB13 (ATCC# PTA-39) in combination with a pharmaceutical carrier.

13. The composition according to claim 12, further comprising an antibiotic.

PATENT COOPERATION TREATY

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To: ROBERT B. MURRAY
ARENT FOX KINTNER PLOTKIN & KAHN, PLLC
1050 CONNECTICUT AVENUE, N.W.
SUITE 600
WASHINGTON, DC 20036-5339

PCT

NOTIFICATION OF TRANSMITTAL OF INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Rule 71.1)

Date of Mailing *(day/month/year)*	17 OCT 2001

Applicant's or agent's file reference	IMPORTANT NOTIFICATION
F108026-00001	

International application No.	International filing date *(day/month/year)*	Priority Date *(day/month/year)*
PCT/US00/06718	12 MAY 2000	12 MAY 1999

Applicant
EXPONENTIAL BIOTHERAPIES, INC.

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices)(Article 39(1))(see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	JAMES HOUSEL
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

Form PCT/IPEA/416 (July 1992) ★

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference F108026-00001	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No. PCT/US00/06718	International filing date *(day/month/year)* 12 MAY 2000	Priority date *(day/month/year)* 12 MAY 1999
International Patent Classification (IPC) or national classification and IPC IPC(7): A01N 63/00; A61K 39/02 and US Cl.: 424/93.6, 234.1		
Applicant EXPONENTIAL BIOTHERAPIES, INC.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 4 sheets.

   ☑ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority. (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 3 sheets.

3. This report contains indications relating to the following items:

   - I [X] Basis of the report
   - II [ ] Priority
   - III [ ] Non-establishment of report with regard to novelty, inventive step or industrial applicability
   - IV [ ] Lack of unity of invention
   - V [X] Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
   - VI [ ] Certain documents cited
   - VII [ ] Certain defects in the international application
   - VIII [ ] Certain observations on the international application

Date of submission of the demand	Date of completion of this report
13 DECEMBER 2000	24 AUGUST 2001
Name and mailing address of the IPEA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer JAMES HOUSEL
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

Form PCT/IPEA/409 (cover sheet) (July 1998) ★

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US00/06718

I. Basis of the report

1. With regard to the **elements** of the international application: *

☐ the international application as originally filed

☒ the description:
pages (See Attached) , as originally filed
pages _____ , filed with the demand
pages _____ , filed with the letter of _____

☒ the claims:
pages (See Attached) , as originally filed
pages _____ , as amended (together with any statement) under Article 19
pages _____ , filed with the demand
pages _____ , filed with the letter of _____

☒ the drawings:
pages (See Attached) , as originally filed
pages _____ , filed with the demand
pages _____ , filed with the letter of _____

☒ the sequence listing part of the description:
pages (See Attached) , as originally filed
pages _____ , filed with the demand
pages _____ , filed with the letter of _____

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.
These elements were available or furnished to this Authority in the following language _____ which is:

☐ the language of a translation furnished for the purposes of international search (under Rule 23.1(b)).

☐ the language of publication of the international application (under Rule 48.3(b)).

☐ the language of the translation furnished for the purposes of international preliminary examination (under Rules 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

☐ contained in the international application in printed form.

☐ filed together with the international application in computer readable form.

☐ furnished subsequently to this Authority in written form.

☐ furnished subsequently to this Authority in computer readable form.

☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

☐ The statement that the information recorded in computer readable form is identical to the writen sequence listing has been furnished.

4. ☒ The amendments have resulted in the cancellation of:

☒ the description, pages NONE

☒ the claims, Nos. NONE

☒ the drawings, sheets/~~fig~~ NONE

5. ☐ This report has been drawn as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).**

** Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17).*

***Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.*

International application No.
PCT/US00/06718

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. statement

Novelty (N)	Claims	1-13	YES
	Claims	NONE	NO
Inventive Step (IS)	Claims	1-13	YES
	Claims	NONE	NO
Industrial Applicability (IA)	Claims	1-13	YES
	Claims	NONE	NO

2. citations and explanations (Rule 70.7)

Claims 1-13 meet the criteria set out in PCT Article 33(2)-(4), because the prior art does not teach or fairly suggest baceteriophages ENB6 and ENB13 that are lytic for susceptible strains for vancomycin-sentitive *Enterococcus faecium*.

---------- NEW CITATIONS ----------

NONE

Supplemental Box
(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: Boxes I - VIII

I. BASIS OF REPORT:

This report has been drawn on the basis of the description,
page(s) 1-13, as originally filed.
page(s) NONE, filed with the demand.
and additional amendments:
NONE

This report has been drawn on the basis of the claims,
page(s) NONE, as originally filed.
page(s) NONE, as amended under Article 19.
page(s) NONE, filed with the demand.
and additional amendments:
Pages 14-16, filed with the letter of 29 July 2001.

This report has been drawn on the basis of the drawings,
page(s) 1-12, as originally filed.
page(s) NONE, filed with the demand.
and additional amendments:
NONE

This report has been drawn on the basis of the sequence listing part of the description:
page(s) NONE, as originally filed.
pages(s) NONE, filed with the demand.
and additional amendments:
NONE